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OZONE UPTAKE IN THE HUMAN NASAL CAVITY:

THE CONTRIBUTION OF URIC ACID

A Thesis in

Chemical Engineering

by

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Abstract

Ozone, a highly reactive, oxidative gas, is the most harmful component of urban smog. The large surface area of the respiratory airway makes it particularly vulnerable to O_3 oxidation. As a protective barrier, a thin coating of mucus blankets the airway surface to prevent direct contact between inhaled toxins and underlying epithelial cells. Furthermore, this lining layer contains an array of biomolecules including antioxidants, which reactively deplete O_3 levels. Previous work has proposed that the antioxidant uric acid (UA) is the major target of O_3 oxidation in the nasal lining fluid (NLF). It has been postulated that O_3 is so reactive that it is depleted before fully penetrating the NLF and contacting epithelial cells. This suggests that harmful effects of O_3 must be mediated by toxic products of O_3 oxidation, such as lipid peroxides and aldehydes.

This work investigated the role of NLF uric acid in modulating O_3 absorption in the human nasal cavity. Ozone absorption was measured as the fractional uptake (Λ) of O_3 from a humidified air stream containing 0.36 parts per million (ppm) O_3, and flowing through the nose unidirectionally at 3 liters per minute (lpm). Contents of the NLF were sampled by nasal lavage with saline. Previous studies report that a relationship between daily measurements of Λ and UA concentration in the NLF ($C_{UA,NLF}$) cannot be observed due to significant day-to-day variations in parameters affecting Λ. To overcome this problem, several perturbations were imposed to induce changes in $C_{UA,NLF}$, and therefore Λ, allowing same-day comparison of these parameters.

In a preliminary study, we investigated the effect of continuous O_3 exposure on Λ in fifteen subjects. It was hypothesized that O_3 exposure would temporarily deplete $C_{UA,NLF}$, resulting in lowered Λ. Results showed that Λ was significantly reduced ($p<0.001$) following exposure to 0.36 ppm O_3 for 30 minutes at 3 lpm. In a subsequent study of twenty-five subjects, we aimed to relate values of Λ and $C_{UA,NLF}$ before and after O_3 exposure. Values of Λ and $C_{UA,NLF}$ were significantly reduced following O_3 exposure and were strongly correlated with each other ($p<0.001$). Regression of these data indicated that $\Lambda=0$ when $C_{UA,NLF}=0$, suggesting a major contribution of UA in modulating O_3 uptake. Reaction-diffusion modeling of the data yielded an apparent second order reaction rate constant between O_3 and UA (k_2) of 1.56×10^9 $M^{-1}s^{-1}$.

In a third study, we investigated effects of the oxidant gas nitrogen dioxide (NO_2) on Λ in twelve subjects. Exposure to 1.0 ppm NO_2 at 3 lpm for 30 minutes resulted in a small reduction of Λ, and no change in C_{UA}. Results of in vitro experiments verified minimal reactivity between gaseous NO_2 and UA in aqueous solution. Although NO_2 is an oxidative gas capable of depleting various NLF compounds, it appears that none of these compounds played a major role in modulating O_3 absorption. Therefore, the facts that 1) $C_{UA,NLF}$ was not influenced by NO_2 exposure and 2) NO_2 exposure induced only a small reduction in Λ, are in agreement with the assumption that UA is the major NLF target of O_3.

Analysis of data from a previous study (Santiago, 2001) that employed serial nasal lavages to dilute UA levels in NLF showed a strong correlation ($p<0.001$) between values of C_{UA} and the corresponding Λ values at each sampling time. The apparent k_2 obtained from this data was 6.04 $x10^8$ $M^{-1}s^{-1}$, which is the same order of magnitude as the value obtained in O_3 exposure studies. However, the results of this analysis indicated that there can be O_3 transport in the absence of UA, likely due to oxidation of exposed cell membranes following serial nasal lavage challenges.

A concentration profile for O_3 in the NLF was simulated with the reaction-diffusion model employing a value for $k_2=10^9$ $M^{-1}s^{-1}$ and $C_{UA,NLF}=200$ μM. Ozone penetration distance into the NLF was estimated to be 0.6 μm, which was less than reported NLF thickness values of 5-10 μm, indicating that O_3 itself cannot not penetrate the NLF to reach underlying cells. Therefore, as a part of the third study, we attempted to detect secondary products of O_3 oxidation, such as lipid peroxides and aldehydes, that might explain how O_3 exposure causes adverse health effects. Results demonstrated significant production of TBARS, a marker of lipid peroxidation, immediately following and 60 minutes following O_3 exposure. Interestingly, increased levels of $C_{UA,NLF}$ appeared to reduce absolute TBARS formation, suggesting a secondary protective role for UA.

Increased cellular production of gaseous nitric oxide (NO) has been implicated during inflammation. Therefore, in the first study, nasal air was monitored before and after O_3 exposure as an indicator of an inflammatory response to O_3. Our data showed a small, but significant elevation of NO one hour following initiation of O_3 exposure, suggesting an emerging inflammatory response.

In summary, we have provided in vivo evidence that UA is the major target of O_3 oxidation in the human nose. The observed value for k_2, and corresponding O_3 penetration depth, provides evidence that O_3 does not reach underlying cells, suggesting that secondary ozonation compounds are responsible for harmful effects of O_3 exposure. In support of this, TBARS levels were significantly elevated following O_3 exposure. Additionally, we show that there may be an inhibitory role of UA in preventing the formation of secondary ozonation compounds. Finally, our results indicate a cellular response to O_3 exposure as measured by NO.

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Chapter 1: Introduction

Ozone (O_3), an oxidative gas, is the major component in urban smog. It is produced by the photochemical reaction involving automobile emissions and atmospheric oxygen. Despite strictly defined regulatory guidelines, successful control of ambient O_3 remains a difficult problem to overcome, as its levels frequently exceed the National Ambient Air Quality Standard (NAAQS) limit of 0.08 ppm averaged over a period of 8 hours.

Ozone is a highly reactive gas capable of oxidizing biologically functional molecules such as proteins, lipids, and antioxidants. Additional harmful effects of ozone arise from its ability to initiate radical formation. Although O_3 exposure can induce systemic toxicity, its effects are primarily isolated to the respiratory system. Studies have shown that O_3 inhalation causes decrements in lung function, induces oxidative stress, and initiates the inflammatory response.

As a form of protection against inhaled toxins, the respiratory tract is coated with a mucus lining layer that provides a physical barrier between the respiratory epithelium and inhaled toxins. Furthermore, this lining fluid contains sacrificial antioxidants that quench the reactivity of oxidative compounds such as O_3. Analysis of nasal lining fluid

has provided evidence suggesting that the antioxidant uric acid (UA) is the major target of ozonation in the nasal cavity.

Ozone uptake, requiring transfer from respired gas to mucous phases, occurs by dissolution followed by rapid reaction with antioxidants. This rapid loss of O_3 limits its concentration in mucus, and thereby maintains a suitable concentration gradient for uptake from respired gas. Rough estimates suggest that reaction of O_3 occurs much faster than diffusion through a typical mucus layer thickness. In other words, O_3 is too reactive to penetrate the lining layer and directly contact epithelial cells. Therefore, it has been proposed that harmful oxidation products of the reaction between O_3 and mucus substrates are ultimately responsible for cellular toxicity.

An important link between environmental O_3 exposure (e.g., exposure duration, concentration) and toxic effect is the dose delivered to target tissue. As a result, studies have investigated factors that influence the distribution of O_3 uptake in the respiratory system, such as gas flowrate, gaseous O_3 concentration, and chemical composition of the mucus layer. The main purpose of this dissertation was to investigate the contribution of UA in the nasal lining fluid (NLF) to O_3 uptake using the nose as a representative model of the respiratory system.

Previous studies have shown that daily measurements of Λ do not correlate with UA concentration due to significant day-to-day variations in Λ. This means that subtle

variations in factors other than UA concentration, such as nasal volume and dietary intake of antioxidants, can mask the observance of a relationship between Λ and UA concentration (C_{UA}). To minimize these confounding effects, we designed and implemented a series of clinical studies to impose perturbations on nasal UA levels in the nose, such that several measurements of Λ could be made at varying levels of C_{UA} for each subject within one day. A reaction-diffusion model was applied to these data to quantify the reaction rate between O_3 and uric acid, allowing us to estimate the penetration depth of O_3 into the mucus layer.

Several molecular endpoints were evaluated as short term stress markers of cellular response to O_3 exposure. Elevated levels of nitric oxide (NO) gas, an indication of inflammation, have been observed in tissues exposed to O_3. Lipid oxidation products, suspected to play a role in O_3 toxicity, confer prolonged tissue damage by sustaining the release of aldehydes and hydroxyl radicals. These compounds can be assayed as thiobarbituric acid reactive substances (TBARS). Therefore, we monitored gaseous NO in nasal air, and TBARS levels in nasal lavage samples as an assessment of O_3 induced damage.

Chapter 2: Background

At high altitudes, the ozone layer serves an essential role in protecting plants and animals from ionizing ultraviolet rays. However, at ground level O_3 is considered an air pollutant. Ozone is a colorless gas resulting from the photochemical reaction between atmospheric oxygen and nitrogen oxides resulting from automobile emissions (Wright et al., 1990).

$$NO_2 + uv \rightarrow NO + O^{\cdot} \quad (2.1)$$

$$O_2 + O^{\cdot} \rightarrow O_3 \quad (2.2)$$

As a major component of urban smog, O_3 is responsible for an array of harmful physiological effects, particularly in the respiratory system (Henderson et al., 1992; Kelly et al., 2003). Ozone levels in urban areas frequently exceed the National Ambient Air Quality Standard of 0.08 parts per million (ppm) averaged over eight hours (Weister et al, 1995). Symptoms of short-term low level exposure are typically reversible and include headache, pain in taking a deep breath and decrements in lung function (Nightingale et al., 1999; Jorres et al., 2000), whereas long-time exposure can adversely affect lung development and can exacerbate existing lung disease, asthma in particular (McDonnell et al, 1999).

Adverse health effects of inhaled ozone arise from its ability to readily oxidize many substrates of the respiratory system including proteins, lipids, and antioxidants (Ballinger et al., 2005, Cross et al., 1994). Studies show that exposure to 0.36-ppm ozone at resting conditions causes significant reductions in forced expired volume in one second (FEV1) (Colucci, 1983). Reductions in FEV1 of 30% were observed following O_3 exposures when healthy subjects exercised moderately. Ozone exposure is known to increase the presence of inflammatory cells such as neutrophils and macrophages in bronchoalveolar lavage fluid (Schelegle et al, 1991). Two-fold increases in total protein, albumin, and immunoglobulin G were found in the lung following exposure to 0.4 PPM O_3 for two hours (Koren et al, 1989), indicating increased epithelial permeability associated with inflammation.

2.1 Ozone uptake in the Nasal Cavity

To impart deleterious effects, inhaled O_3 must first absorb into tissue. Consequently, understanding the factors influencing respiratory O_3 uptake would shed light on its toxic mechanisms in the airways. Weister (1996) reported an inverse relationship between minute volume and O_3 fraction retained in the lungs. Rigas et al (1997) demonstrated that continuous O_3 exposure reduces subsequent O_3 uptake in the lung, whereas exposure to the pollutant nitrogen dioxide may augment O_3 uptake. Santiago et

al (2001) showed that uptake in the nose was correlated with gas flow rate and antioxidant concentration.

We will focus our attention on O_3 uptake in the nasal cavity for several reasons. During nasal breathing, the nasal airways represent the first contact between inspired air and the respiratory system. As a result, tissues lining the nasal cavities experience the highest pollutant concentration in the respiratory system. From an experimental standpoint, the nasal cavity provides an easily accessible surrogate for investigation of uptake in the respiratory system as a whole. There is also an advantage in terms of safety; by restricting the investigation of O_3 uptake and toxicity to the nasal cavity, the lungs are spared from oxidative damage. In addition, nasal lining fluid (NLF) constituents are more easily sampled by performing nasal lavage rather than sampling lung lining fluids via bronchoalveolar lavage (BAL), which requires a subject to undergo anesthesia (Mudway et al., 1999)

Another advantage of studying O_3 uptake in the nose is that, during nasal breathing, a large fraction of the total uptake is isolated to the nasal airways. O_3 bolus penetration studies in human lungs show that during a full breath at resting conditions, between 80-90% of inspired O_3 is absorbed in the nose (Nodelman and Ultman, 1999). Santiago et al. (2001) demonstrated about 50% absorption of O_3 in the nose, from a unidirectional stream of ozonated air introduced into one nostril at a constant volumetric flow corresponding to a resting ventilation rate of 8 lpm.

Because much of the total O_3 uptake occurs in the nasal cavity, in spite of its small volume, the nose serves an important role in protecting distal airways from exposure (Calderon-Garciduneas et al. 1998). However, this protection comes at the expense of antioxidants and sacrificial targets of O_3 in the fluid lining the upper airways (Aslplund et al. 1996, Avissar et al., 2000).

2.2 Respiratory Lining Fluid, Antioxidants, and Mechanisms of Ozone Toxicity

The respiratory tract lining fluid (RTLF) provides an aqueous physical barrier between inspired air, and epithelial cells. RTLF thickness varies in different parts of the airways. For example, thickness ranges from 5-10 μm in the nasal cavity, but is much thinner in the distal lung at 0.2-0.5 μm (Hatch, 1992). The RTLF is composed of two aqueous layers (Kaliner et al, 1991); 1) the sol layer: a low viscosity layer directly lining epithelial cells, and 2) the gel layer: higher viscosity layer blanketing the sol layer and directly interfacing with the airway. The respiratory epithelium is ciliated, resulting in a net movement of the lining fluid, particularly the gel layer, towards the oropharynx at about 1 cm/min .

In addition to providing a physical diffusion barrier against inhaled toxins, a variety of RTLF compounds chemically quench the toxicity of absorbed agents (Cross et al, 1994; Hatch, 1992). Oxidation targets of O_3 include lipids, proteins, and antioxidants, with

antioxidants being the most reactive substrate (Uppu et al., 1995). Because lipid and protein molecules provide biochemical function, oxidative damage of these entities may confer toxic effects. On the other hand, antioxidant molecules generally serve a sacrificial role, and their oxidation has little direct impact on cellular function. Therefore, it can be hypothesized that higher levels of RTLF antioxidants provide added protection against oxidant attack. However, during oxidative stress, antioxidant levels may not be sufficient to absorb the oxidant burden, allowing oxidation of other compounds.

Several studies have demonstrated significant correlations between increased antioxidant levels and reduced toxic effects of inhaled O_3. Pryor (1991) suggests that supplementation of the antioxidant α-tocopherol above the minimum dietary requirement increases systemic protection against biochemical effects of O_3. Romieu et al. (1998) showed that long term supplementation of the antioxidants ascorbic acid, α-tocopherol, and β-carotene reduced decrements in FVC, FEV1, and FEF_{25-75} in street workers residing in Mexico City, a city that experiences particularly high levels of ambient O_3. Mudway (1998) demonstrated that albumin was spared O_3 oxidation in antioxidant solutions, but underwent significant oxidation in antioxidant-free solutions. Ballinger et al (2005) provides evidence that at low concentrations, the antioxidant ascorbic acid may actually promote lipid and membrane oxidation, whereas these effects are not observed at higher levels. In a controlled O_3 exposure study, Samet et al

(2001) found that high intake of ascorbate was significantly correlated with reduced decrements in lung function associated following exposure.

The low molecular weight antioxidants uric acid (UA), ascorbic acid (AH_2), and glutathione (GSH) have been implicated as the most preferred targets of O_3 attack in the RTLF (Cross et al, 1994). These compounds exist at varying concentrations throughout the respiratory system. For example, ascorbic acid concentration ranges from 100-400 μM in the lung lining layer (LLF), but is only 40 μM in the nasal lining fluid (NLF). The reactivity of O_3 with these compounds also varies, with ascorbic acid having the highest rate constant. Table 2.1 compares regional antioxidant concentrations in NLF and LLF and bimolecular rate constants determined from studies in antioxidant solutions (Cross et al, 1994; Mudway and Kelly, 2000).

Antioxidant	NLF, μM	LLF, μM	k_2, $M^{-1}s^{-1}$
Uric Acid	100-400	90	$1.4x10^6$
Ascorbic Acid	10-50	100	$6x10^7$
Glutathione	40[1], <10[2]	100	$2.5x10^6$

Table 2.1. Antioxidant concentrations in NLF and LLF, and second order rate constants. [1]Cross et al, 1994. [2]Mudway and Kelly, 2000.

Although it appears that ascorbic acid is the most susceptible antioxidant target for O_3 oxidation, uric acid probably contributes more to O_3 removal in the nasal cavity. Peden

et al. (1990, 1991) inferred that UA plays a major role in quenching O_3 by demonstrating that it is the only stable low molecular weight antioxidant in nasal secretions. Mudway and Kelly (1998) demonstrated that in the presence of protein, UA promotes O_3 removal at a greater rate than that of AH_2 and GSH combined. With respect to reaction with O_3 in the nose, differences between the relative importance of UA, AA, and GSH may be a result of their excretion mechanisms as well as their reaction rates. RTLF concentrations of AA and GSH are thought to be in equilibrium with their respective concentrations in the plasma. Therefore, AA and GSH transport to the RTLF requires mass transfer by passive diffusion across the respiratory epithelium. On the other hand, UA is known to be stored locally in nasal epithelial mucosal cells. Consequently, UA is capable of rapid release, whereas AA and GSH are not (Peden et al., 1993).

Because O_3 is only sparingly soluble in aqueous solution, it has been postulated that its uptake into the respiratory system is driven by a reaction-diffusion mechanism (Langford et al., 1995). In this process, O_3 absorbed into the aqueous phase is immediately scavenged by antioxidants, thus maintaining a favorable concentration gradient for rapid absorption into the RTLF. Consequently, upon exposure, O_3 is postulated to exist only within a thin film at the RTLF-airway interface, and it is assumed that underlying epithelial cells are spared from direct ozonation at the cost of RTLF antioxidants. A study conducted by Hatch et al (1995) supports this assertion by

illustrating that O^{18}- labeled O_3 administered to rats was isolated to the extracellular space, rather than within cells.

Studies have demonstrated reduced UA levels following exposure of artificial solutions and lavage samples to O_3. However, thus far, only Mudway et al. (1999) have investigated UA in response to acute O_3 exposure in the human nasal cavity. Their work verified that continuous nasal exposure to 0.2 ppm for one hour results in significantly reduced UA levels as indicated by nasal lavage.

Because it is assumed that O_3 does not fully penetrate through the RTLF to oxidize cells directly, it has been theorized that its toxic effects originate instead from a cascade of oxidation products formed by reaction between O_3 and RTLF substrates. Lipid ozonation products result from the reaction between O_3 and unsaturated fatty acids (UFA) (Giamvala et al. 1985). These products are suspected to be the main carriers of O_3 toxicity due to the abundant presence of UFA in the RTLF, and because lipid ozonation products are relatively stable and diffusive (Pryor et al, 1995). In addition, Kafoury et al (1999) demonstrated that exposure to lipid ozonation products initiates an inflammatory response similar to that arising solely from O_3 exposure, indicating a common origin of toxicity.

Specifically, it is believed that the main sources of O_3 toxicity are lipid hydroxyhydroperoxides and aldehydes. These toxic compounds are produced by the

reaction between O_3 and C=C bonds of unsaturated fatty acids (UFA), such as phosphatidycholine, which constitutes about 70% of total lipids found in the RTLF. In the absence of water, the sole product of this reaction is the Criegee Ozonide. However, in aqueous solution, this pathway only constitutes 10% of total reaction, and the bulk of products formed are aldehydes and lipid peroxides. Lipid peroxides confer further toxicity, breaking down to form an aldehyde (RHCO) and hydrogen peroxide (HOOH). Hydrogen peroxides and aldehydes have well characterized toxic effects. Hydrogen peroxide is particularly toxic in that it readily decomposes to form hydroxyl radicals ($^{\bullet}OH$), which may initiate a radical chain reaction with many biological substrates, including lipids and proteins (Pryor, 1994). Furthermore, $^{\bullet}OH$ is capable of compromising antioxidant defenses by direct reaction (Ames, 1981; Doba, 1985). The early cascade chemistry of UFA ozonation is presented in figure 2.1 (Pryor et al., 1995).

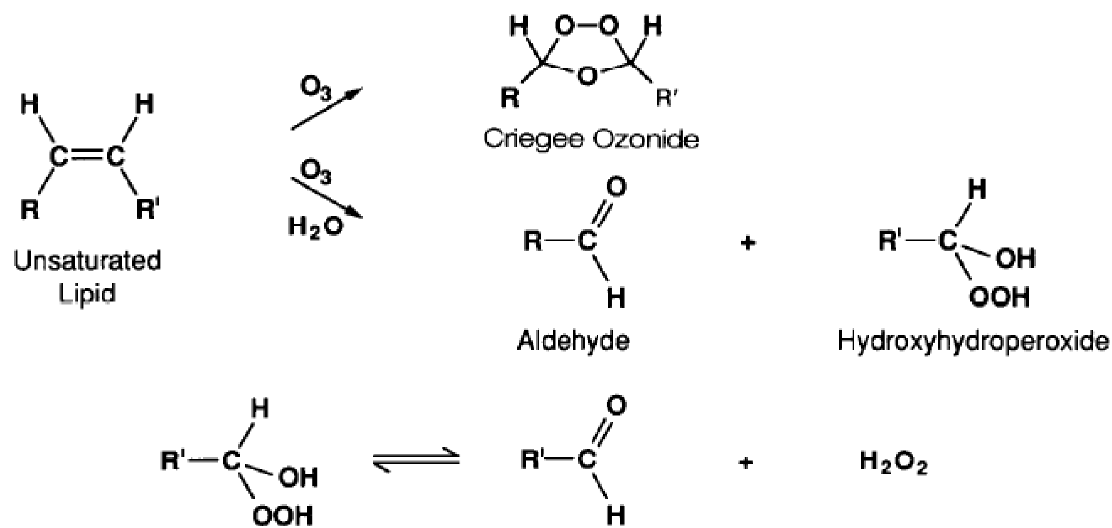


Figure 2.1. Mechanism of formation of lipid ozonation products.

Several studies have investigated O_3 induced lipid peroxidation in vitro and in vivo. In a red cell membrane model, Ballinger et al. (2005) demonstrated that artificial solutions with AH_2 levels below 200 μM exhibit a closely linear relationship between AH_2 and lipid peroxidation, indicating a pro-oxidant nature for AH_2. However, AH_2 appears to inhibit lipid peroxidation at higher concentrations (>200 μM). Long et al (2001) showed an O_3 dose dependent increase in lipid peroxidation following exposure in a hamster model.

It must be noted that proteins also undergo O_3 oxidation to generate a cascade of free radial reactions whose products are capable of inducing toxicity. However, due to the low concentration of proteins relative to lipids in the RTLF, and due to the larger size of their oxidation products, toxic effects of protein oxidation are generally not as appreciated as those arising from lipid oxidation. Additionally, Mudway and Kelly (1998) showed minimal oxidation of protein in model solutions.

2.3 Toxic Effects of Nitrogen Dioxide Exposure

Because of its underlying mechanism of formation (2.1), atmospheric O_3 is commonly accompanied by nitrogen dioxide (NO_2), another oxidative air pollutant. Like O_3, NO_2 rapidly oxidizes biological compounds such as proteins and lipids (Ben-Jebria et al. 1998; Velsor et al, 2003). Acute airway exposure to low levels of NO_2 induces oxidative stress, lesions in respiratory epithelium, and inflammation (Connor et al.,

2001). Brief exposures to NO_2 have been shown to increase the severity of allergic inflammation in asthmatics (Barck et al, 2004). The 8-hour time-weighted average limit for NO_2 is 3 ppm as defined by the American Conference of Governmental Industrial Hygienists. The NAAQS sets the yearly averaged limit on ambient NO_2 levels much lower at 0.53 ppm. However, ambient outdoor NO_2 levels have been well within control limits since the mid 1980's.

An important aspect of NO_2 exposure with regard to this study is that it has been implicated as a potentiating agent for the effects of O_3 exposure. Mustafa et al (1984) showed that exposure to O_3 or NO_2 alone did not induce biochemical changes in rat lung, but changes in several parameters were more than additive when the gases were administered together, indicating a synergistic effect. Denicola et al (1981) demonstrated that NO_2 exposure results in dose–dependent enhancement of tissue permeability assessed by vascular protein exudation in a hamster model. Rigas et al. (1997) showed significantly increased uptake of O_3 in the human lung following exposure to 0.36 ppm NO_2, but not 0.72 ppm. Therefore, these studies suggest that NO_2 may have a priming effect for O_3 toxicity.

2.4 Inflammatory Response to O_3 Exposure

Exposure to inhaled O_3 activates the immune response, resulting in inflammation of the airways. The degree of inflammation has been previously assessed by measuring

molecular endpoints, such as interleukins and immunoglobulins (Jorres et al.,2000), or by quantifying inflammatory cell recruitment and activation such as macrophages (Koike et al., 1998) and neutrophils (Schelegle et al., 1991), at the site of exposure.

It has been proposed that increased levels of exhaled nitrogen oxide (NO) may be released in response to inflammation (Zapol et al., 1994). Nitric oxide is synthesized in cells by a group of enzymes known as nitric oxide synthases (NOS). Three NOS isoforms exist: 1) endothelial, 2) neuronal, and 3) inducible. Endothelial and neuronal isoforms are basally active in endothelial cells and neurons, respectively. As a result, these tissues constantly produce NO (Kharitonov and Barnes, 1996). The third isoform, inducible NOS (iNOS), is not basally active in cells. iNOS synthesis occurs only during an inflammatory response in endothelial and inflammatory cells. As a result, these cells exhibit elevated NO production during inflammation. (Quinn et al, 1995).

Nitric oxide is synthesized from the amino acid L-arginine in the presence of oxygen by any of the above-mentioned isoforms. The products of this reaction are L-citrulline and NO. The production pathway for NO is found in figure 2.2

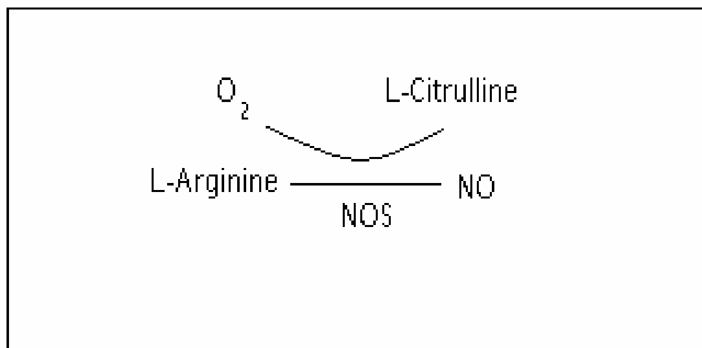


Figure 2.2 Production Pathway for NO

Nitric oxide is found in gaseous form in the respiratory system, and therefore can be readily sampled and monitored in exhaled air by chemiluminescent detection via reaction with O_3 (Hogman et al, 2000; Lundberg and Weitzberg, 1999). Typical NO concentration in the lungs ranges from 5-10 parts per billion (ppb), and is about 100 ppb in the nasal cavity. Significantly large amounts of NO (up to 20 ppm) are found in the sinuses (Haight et al, 1999). This is most likely due to accumulation of NO in the poorly ventilated the sinus spaces.

The relationship between inflammation and exhaled NO has been documented empirically. Patients with asthma, an inflammatory disease, display increased NO levels in exhaled air compared to non-asthmatics (Kharitonov et al, 1996). Similarly, Sato et al. (1998) and Garrelds et al. (1995) reported increased nitric oxide metabolite concentrations in nasal lavage of patients with allergy induced inflammation. Several studies have aimed to discover a relationship between inhaled O_3 and NO and its mediators. Punjabi et al (1994) demonstrated that O_3 exposure stimulated an increase

in NO levels, measured as aqueous nitrate and nitrite, in type II pulmonary cells of rat lungs. This increase in nitrate and nitrite correlated with increased expression of iNOS mRNA and iNOS protein (Pendino et al, 1996). However, these studies did not aim to measure NO in expired air. Olin et al (2001) investigated exhaled NO in response to short term O_3 exposure, though no significant changes in exhaled NO were observed. On the other hand, pulp mill workers, who were occupationally exposed to ozone, had higher exhaled NO levels compared to a non-exposed population (Olin et al, 1999).

Nitric oxide is very reactive with O_3, and therefore may act as an airway antioxidant. This effect is probably minimal, however (Santiago et al, 2001).

2.5. Nasal Airway Geometry

During nasal breathing, the nasal cavity acts as the first surface of contact between the external environment and the respiratory system. Nasal volume typically ranges from 20-30 cm^3 and provides a surface area of up to 200-300 cm^2 (Guilmette et al., 1989). This high surface area is responsible for providing temperature and humidity conditioning of inspired air before reaching the lungs.

A septum divides the nasal cavities into two halves, each with its own airway opening (nostril). From each nostril, air passes through a nasal valve, which has the lowest cross sectional area throughout the nasal cavity. Immediately downstream of the nasal

valve in the antrum, each air stream is partitioned into three partial channels by the turbinates, structures protruding laterally towards the septum. The turbinates provide added surface area for exchange between the gas and RTLF phases. After traversing the turbinates, the two airstreams that were initially separated at the nostrils rejoin in the nasopharynx and proceed to the throat and lungs. A schematic of the nasal cavity is given in figure 2.3.

The nasal cavity has a highly vascularized structure. As a result, disturbance to the nasal epithelium may induce variations in blood flow, thereby altering surface geometry and nasal cavity volume. This can be brought on by swelling due to changes in temperature, sympathetic activity, or inflammation. In addition, changes in nasal volume may occur due to nasal cycling, which is characterized by natural periodic changes in nasal volume. Cyclic changes in nasal volume are generally out of phase between left and right sides of the nose. The exact purpose of this periodic swelling is unknown. However, it has been proposed that swelling facilitates pumping of plasma components to the NLF (Eccles, 1996).

Because nasal volume is variable in nature, knowledge of its behavior is essential when measuring parameters that may be volume or surface dependent, such as O_3 uptake and NO production.

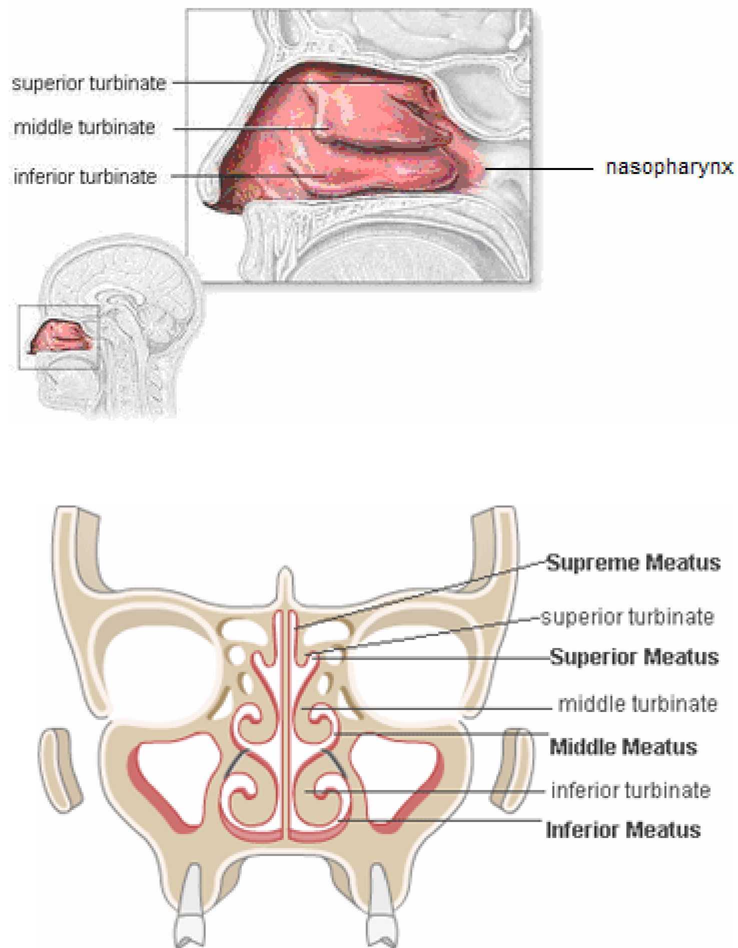


Figure 2.3 Diagrams of the nasal airway. Top: Lateral view of the nasal cavity. Bottom: Front view of the nasal airways. Dark regions indicate airways. Images courtesy of The Empty Nose Syndrome Association.

Chapter 3: Materials and Methods

The technical elements for this study are illustrated in this chapter. A description of various equipment and methods used to obtain data, both directly from human subjects and indirectly from nasal lavage samples, is provided. This is followed by an explanation of procedures followed during experimental sessions performed on human subjects. Finally, a discussion of the mathematical diffusion-reaction model used to analyze the O_3 absorption data is included.

3.1 Maneuvers and Measurements on Human Subjects

3.1.1 Measurement of Ozone Uptake in the Nasal Cavity

The fractional absorption of O_3 (Λ) was measured during unidirectional flow of air through the nasal cavity. A humidified air stream, carrying 0.36 parts per million (ppm) O_3, was introduced into one nostril at 3 liters per minute (lpm) and circulated through the nasal cavity towards the opposite nostril. Upon exiting this nostril, a fraction of the flow was sampled by an O_3 analyzer. To facilitate delivery and recapture of the gas streams to and from the nasal cavity, nostrils were fitted with 1 cm diameter ground glass joint balls that the subject held against each nostril (Joint Ball

18/7 Kimble Kontes, Vineland, NJ). Glass joint balls were adapted to Teflon tubing using flexible tygon tubing and Teflon tape.

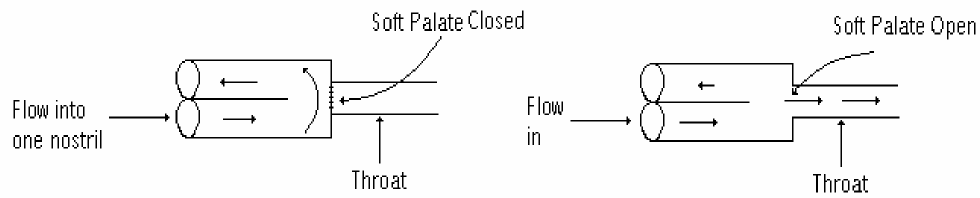


Figure 3.1. Schematic of the nasal airways demonstrating the effect of soft palate position on nasal flow pattern during unidirectional flow. Upon soft palate closure, the flow introduced to one nostril is fully directed towards the exit nostril (left). An open or partially open soft palate allows a fraction of the introduced stream to leak to the lower airways (right).

To ensure the nasal cavity was isolated from lower airways, the soft palate was closed to occlude the distal portion of the nasopharynx as shown in figure 3.1. Soft palate closure was achieved according to the American Thoracic Society (ATS) standard by creating an oral pressure of 5-10 cm H_2O by asking the subject to expire orally through a mechanical flow resistor (ATS board of directors, 1999). To achieve this standard, a mouthpiece was connected to one leg of a tee via Tygon tubing. The remaining two legs of the tee were connected to a pipette tip installed in one end to provide a high resistance to flow and a manometer to display real-time oral pressure (Fig. 3.2). The targeted pressure range was marked on the manometer, and provided visual feedback to the subject. Nasal air sampling was performed during this expiratory maneuver, which lasted 15 seconds.

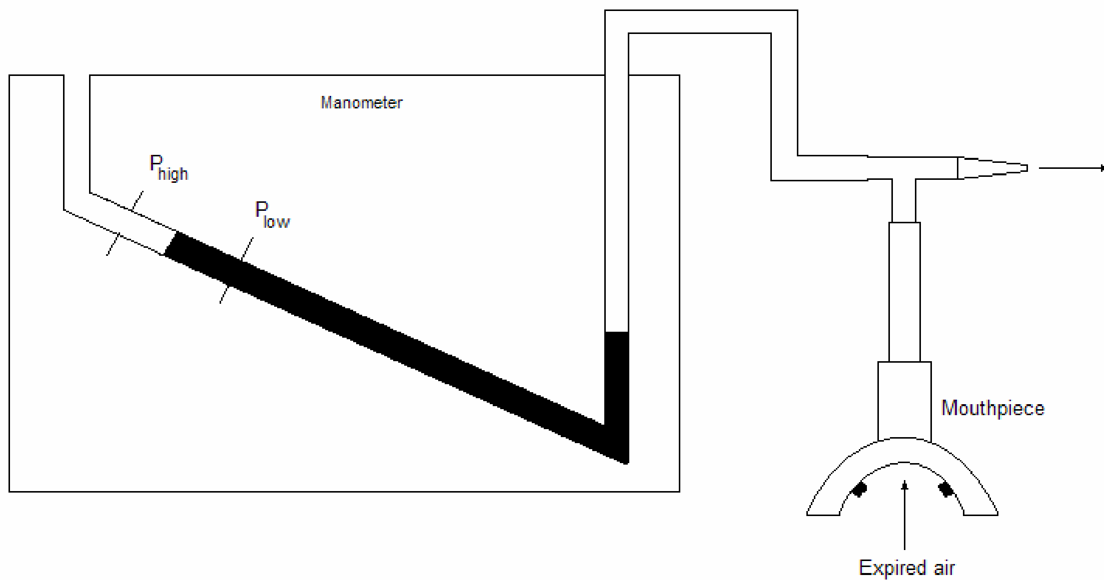


Figure 3.2. Illustration of oral pressure generation assembly. Subjects exhaled orally into the mouthpiece. Resistance to flow was provided by a pipette tip attached to one leg of the tee. Real time pressure, as indicated by the meniscus of the fluid, was viewed by the subject who adjusted his exhaled flow accordingly. The acceptable range of pressure was indicated on the manometer as P_{high} and P_{low}, in this case 10 cm H_2O and 5 cm H_2O, respectively.

The nasal glass joint ball assembly and associated tubing carrying O_3 were connected to a control box equipped with two two-way solenoid valves. Each solenoid valve had three ports: common, normally open, and normally closed. Valve positions were coordinated by a computer program that controlled sampling of three separate streams: nasal input, nasal output, and room air. A diagram of the ozone uptake assembly is shown in figure 3.3.

Ozone detection was based on the chemiluminescent reaction between sampled ozone and a source of pure ethylene (MacDougal et al., 1998; Santiago, 2001). Two gas streams simultaneously entered the detection chamber of the O_3 analyzer: 1) pure ethylene flowing at 2.4 lpm, and 2) an O_3 sample stream flowing at 600 mL/min. The chemiluminescence was measured by a photomultiplier tube. A two-point calibration of the instrument was performed at 0 and 1 ppm. During uptake measurement, gas streams were sampled in the following order: nasal inlet, room air, and nasal outlet. A typical output for nasal ozone uptake is displayed in figure 3.4.

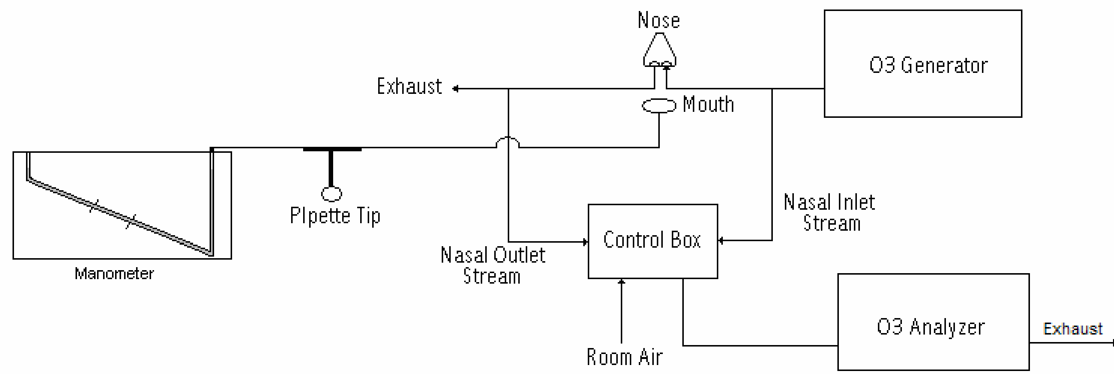


Figure 3.3. Diagram of the flow scheme for measurement of nasal ozone uptake.

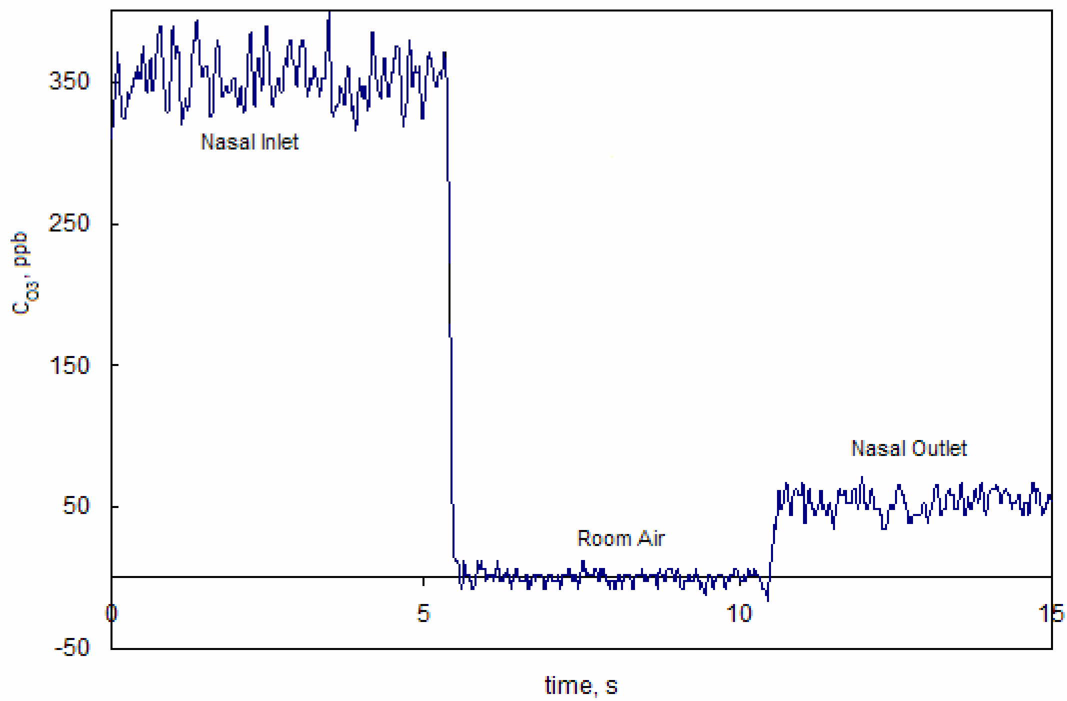


Figure 3.4. Ozone analyzer output. Each indicated stream was sampled for 5 seconds. The fractional ozone uptake value for this maneuver was 0.806.

Fractional ozone uptake, Λ, was calculated as,

$$\Lambda = (1 - C_{out}/C_{in}) \qquad (3.1)$$

where C_{out} and C_{in} represent nasal outlet and nasal inlet ozone concentrations, respectively. Each plateau on the output was averaged by a computer program to determine the corresponding stream's average O_3 concentration.

Ozone was generated by passing clean air across an ultraviolet (UV) lamp, converting molecular oxygen into ozone. Ozone concentration was adjusted by manually varying voltage supplied to the UV lamp.

3.1.2 Determination of Nasal Nitric Oxide Production

This measurement allowed quantification of gaseous NO concentration (C_{NO}) exiting the nasal cavity at a fixed flow rate. Clean humidified air was introduced into one nostril at 3 liters per minute (lpm) through a glass joint ball, and circulated through the nasal cavity while sweeping nitric oxide towards the opposite nostril (Djupesland et al, 2001). A glass joint ball at the exit nostril was equipped with a tee that partitioned the stream in two: 1) one stream was sampled by a NO analyzer at 200 ml/min, and 2) the remaining portion was vented to the room. All C_{NO} measurements were performed during oral exhalation against a resistance (Figure 3.2) to ensure that all NO originating

from the nasal cavity was accounted for. This maneuver lasted for 20 seconds. Because all measurements were taken at the same flow rate of 3 lpm, the NO production rate was directly related to C_{NO}, and therefore will be reported as such.

A Nitric Oxide Analyzer (Sievers model 280, Manchester, UK) was used for quantification of NO. Its method of detection was based on the chemiluminescent reaction between sampled nitric oxide, and supplied ozone. A two-point calibration of the instrument was performed prior to each use at 0 and 5 ppm.

The instrument response typically achieved a stable signal within 5 seconds. NO levels indicated by the instrument are represented by [NO]. Steady state averaged NO concentrations, C_{NO}, were determined by averaging instrument output that was obtained between 10 and 20 seconds after the start of NO data collection. Figure 3.5 displays a typical [NO] profile.

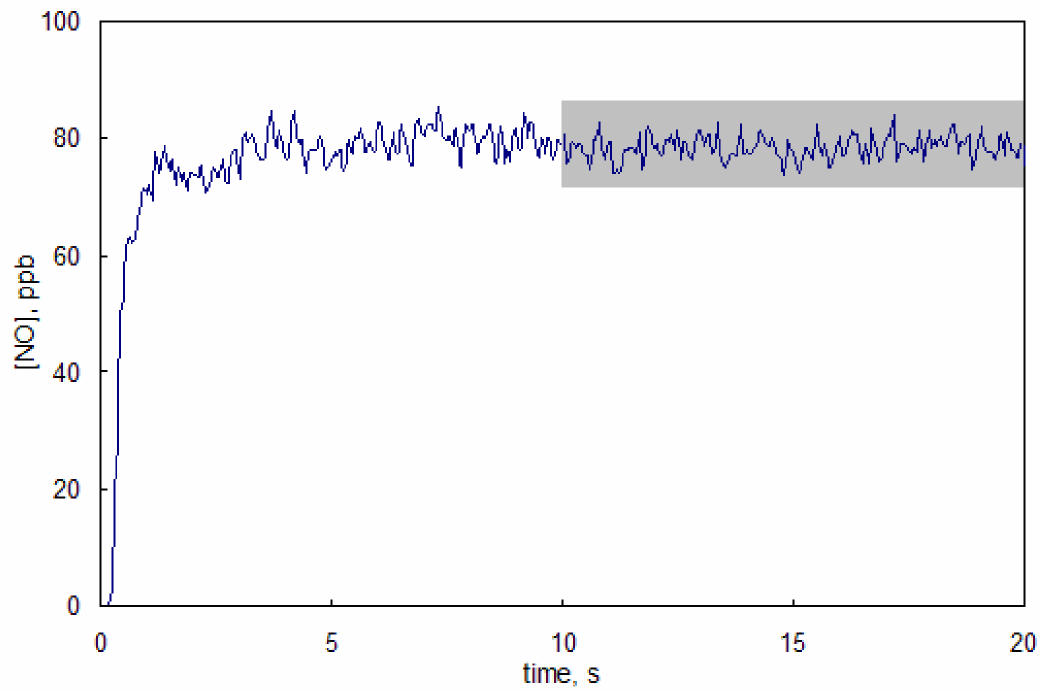


Figure 3.5. NO analyzer output. The NO output was averaged from 10-20 seconds to obtain a steady state averaged concentration, C_{NO}, of 78.6.

3.1.3 Nasal Ozone Exposure Assembly

Three exposure gases were used in this study: 1) a pure air stream, 2) an air stream containing 0.36 ppm O_3, and 3) an air stream containing 1 ppm NO_2. All exposure streams were humidified prior to addition of O_3 or NO_2 by sparging through sterile water. The source of air for all exposure streams was provided by a compressed zero-air cylinder.

The O_3 exposure stream was generated by passing clean air across an ultraviolet (UV) lamp, thus converting a fraction of oxygen molecules to ozone. Output O_3 concentration was controlled by manually adjusting voltage supplied to the lamp. The nitrogen dioxide exposure stream was produced by passing air through a permeation chamber (Dynacalibrator Permeation Chamber, Valco Instruments Co., Poulsbo, WA). A certified permeation tube containing liquefied NO_2 was placed inside the permeation chamber. Target NO_2 levels were achieved by setting the chamber temperature and air flow rates as prescribed by the calibration supplied by the manufacturer of the permeation tube.

The flow scheme for nasal exposure was such that a parallel stream of desired gas was applied to both nostrils in the inspiratory direction through glass joint balls. Because

the flow was continuously introduced into both nostrils in the inspiratory direction, subjects were instructed to breathe orally during exposure.

3.1.4 Determination of Nasal Volume by Acoustic Rhinometry

An acoustic rhinometer (Eccovision, E. Benson Hood Laboratories, Pembroke, MA) was used to determine nasal volume. For this measurement a wave tube generated sound impulses that were introduced into the nasal cavity through a nosepiece that the subject sealed against one nostril. Sound impulses reflected throughout the nasal cavity and incident sound waves were recorded by a microphone inside the wave tube. A computer program converted these acoustic input-output data into a rhinogram; a profile of cross sectional area as a function of distance from the nostril opening. Rhinograms were numerically integrated to yield nasal volume. This measurement was performed twice for each nostril. Nasal volumes obtained for each nostril were added to determine total nasal volume. A sample rhinogram is shown in figure 3.6. The instrument was calibrated daily by a cylindrical calibration tube of known cross section and volume.

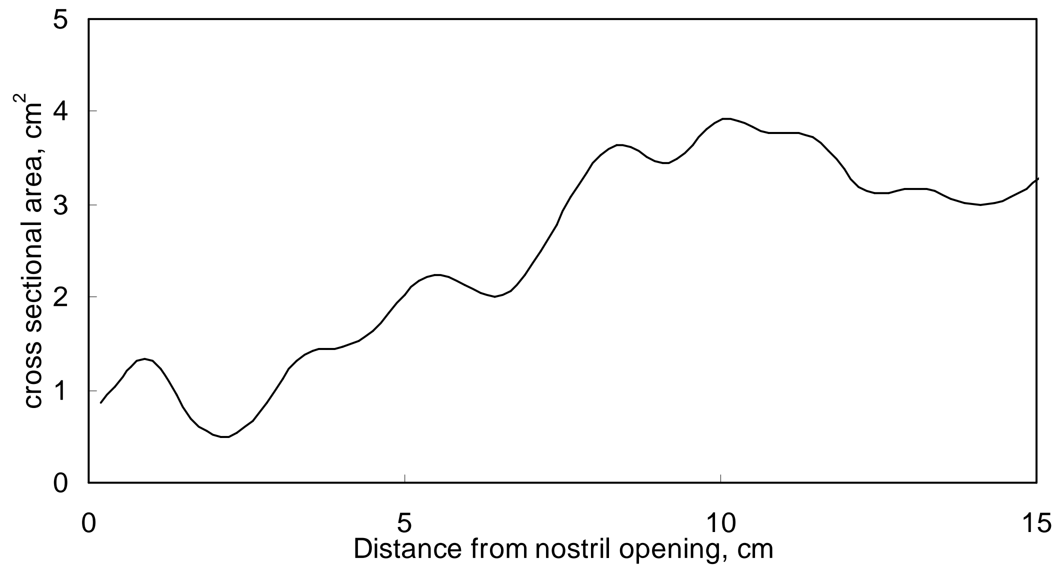


Figure 3.6. Rhinogram for one nostril. Integration of this profile yielded a nasal volume of 28.3 ml.

3.1.5 Nasal Lavage Procedure for Collection of NLF Constituents

Nasal lining layer constituents were sampled by instilling five milliliters of sterile isotonic saline into one nostril using a pipette or syringe. Subjects were instructed to tilt their heads back while holding their breath, thereby raising their soft palate and preventing swallowing of the saline. After 10 seconds, subjects leaned forward and their nasal contents were drained into a sterile specimen cup. This was repeated for the opposite nostril, draining the second sample into the same cup. Particulate matter was removed from samples by passing through a 0.45 μm filter (Gelman Sciences, Ann Arbor, MI). The filtered sample was stored at -80°C and later analyzed for various compounds.

3.1.6 Pulmonary Function Testing

Lung function was assessed using a spirometer (CDX, Providence, RI). The instrument was calibrated with a 3-liter gas-tight syringe prior to each use. After calibration, subjects were instructed to inhale room air to full lung capacity, and forcibly exhale as much air as they possibly could though the mouthpiece of the instrument. Flowrate was measured by a pneumotach inside the system and recorded as a function of time. Subjects repeated the measurement until two matching flow-time profiles were obtained. The instrument provided standard lung function parameters such as forced vital capacity (FVC) and forced expired volume in one second (FEV1).

3.2 Analysis of Components in Nasal Lavage

3.2.1 Quantification of Uric Acid by HPLC

Lavage samples were separated using a reverse phase high performance liquid chromatograph (Model HP1100, Agilent Technologies, Palo Alto, CA). For each sample, 100 μL of lavage sample was injected into the column (Supelcosil LC-18, particle size = 5 μm, Supelco, Bellefonte, PA). Components were separated based on polarity, with more polar compounds eluting faster. The mobile phase for uric acid separation consisted of a buffered (pH=2.1) solution of 2M KH_2PO_4 which passed through the column at 1 mL/min. At these operating conditions, the retention time for uric acid in the column was 3.4 min.

Separated bands were analyzed by an electrochemical detector (INTRO, GBC Separations, Hubbardston, MA) that generated current upon detection of uric acid. The electrochemical detector contained an Ag+/AgCl reference electrode with cell voltage set to +0.8 V. Current output from the detector was recorded as a function of time by a computer program that generated a peak for each separated band. Peak identity was determined by comparing retention time with those obtained from standard solutions. Concentrations were determined by directly comparing area under the curve with those obtained from a standard curve. Each sample was analyzed in triplicate.

3.2.2 Analysis of Total Protein Levels

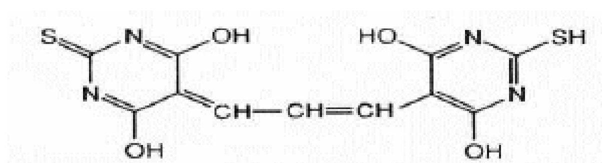
Total protein concentration in lavage samples was determined using commercially-available spectrophotometric assay kits (Pierce, 23200; Bio-Rad, 500-0001). These assays were based on the Bradford method for analysis of protein concentration. This involved the addition of the acidic dye, coomassie blue, to the protein solutions. Coomassie blue readily binds to basic and aromatic amino acid residues, shifting the absorbance maximum of the dye from 465 nm to 595 nm.

Bovine serum albumin was initially used as the standard for total protein analysis. Gamma globulin was used as the standard in later tests because it was reported that it is present in respiratory tract lining fluid. Reports indicate that similar levels of albumin and gamma globulin are present in the RTLF. Standard solutions ranged in concentration between 0-25 μg/mL. Standard curves for these two compounds were not identical. However, ranges of total protein levels were similar for both tests. Additionally, human subject protocols were designed to monitor changes in total protein levels throughout exposure sessions, rather than determine absolute values. In early studies, nasal lavage samples were diluted 12 fold to bring protein concentrations to within the range of the standard curves; however, in later studies lavage samples were diluted 23 fold. Each sample was treated with the coomassie blue solution and incubated at room temperature for 5 minutes, then screened for absorbance at 595 nm.

Protein concentrations were determined by using Beer's law and by comparing absorbance of unknown samples to those obtained by from a standard curve.

3.2.3 Determination of TBARS Levels

The degree of lipid peroxidation was assessed using an assay that indicates the level of thiobarbituric acid reactive substances (TBARS). Biological samples contain a mixture of compounds that react with thiobarbituric acid (TBA), including lipid peroxides and aldehydes. Although a few substances are known to interfere with this assay that are not specific to lipid peroxidation, the TBARS assay remains the most widely accepted method for lipid peroxidation assessment. The standard curve for this assay was based on the addition reaction between malondialdehyde (MDA) and thiobarbituric acid, resulting in formation of a 1:2 adduct between these compounds shown below.



The concentration of this compound can be measured by fluorometry or spectrophotometry, with the former being the more sensitive method. In this work, TBARS analysis was performed using the fluorometric method.

The range of the standard curve was 0-4 nmol/L. Samples were mixed in test tubes with SDS solution and TBA reagent as indicated by the assay (TBARS Assay Kit, Catalog # 0801192, Zeptometrix Corporation). The mixtures were then incubated for 60 minutes at 95°C in an oven or water bath. Following incubation, samples were immediately cooled in an ice bath. Samples were read by a fluorometer with excitation and emission wavelengths of 530 and 550 nm, respectively. Slit width was set to 5 nm for excitation and emission beams.

3.3 Experimental Design

This section describes experimental protocol and safeguards employed for human subject experiments.

3.3.1 Human Subject Safety and Screening

All study protocols were launched following Penn State Institutional Review Board (IRB) review and approval. Experiments involving human subjects were performed under close supervision of The General Clinical Research Center (GCRC) nursing staff, with a medical doctor or nurse practitioner present in-house during all procedures. Non-smoking male and female subjects age 18-32 were recruited by posting flyers on the PSU campus (Appendix A). Subjects first met with the investigator for an introduction to procedures, and to qualify their eligibility for the study. Subjects completed several questionnaires for assessment of medical and smoking history, and

signed a consent form describing the study (Appendix A). Subjects were excluded from participation if they had smoked within three years of participation. Pulmonary function parameters were measured by spirometry to assess lung function. A FEV1/FVC ratio of at least 0.7 was required for participation. Subjects with no history of respiratory or cardiovascular conditions that were not taking prescription medication (excluding birth control medicine) were scheduled for a physical examination by a clinician. Female subjects underwent a urine hCG test to determine pregnancy status and were excluded from participation if results were positive. Following successful evaluation by the clinician, subjects were cleared to participate in the study.

Vital signs were measured by a nurse before and after all research sessions. Female subjects were given a urine hCG test immediately prior to all procedures to ensure they were not pregnant at the time of the experiment.

3.3.2 Protocol AF1: Pilot Study – Determination of the effect of continuous nasal O_3 exposure on subsequent O_3 uptake and NO production

This study was designed as an initial probe to determine the effect of continuous O_3 exposure on the responses of Λ and C_{NO}. It was hypothesized that continuous contact between the NLF and O_3 would result in reactive depletion of resident antioxidants, thus reducing Λ. Over time, a state of reduced antioxidant defenses would allow tissue oxidation to occur, thus resulting in inflammation and augmented NO production by underlying tissue.

Each subject participated in two experimental sessions. Both sessions followed the same procedures, with the only exception being the exposure gas used. In the first session subjects' nasal cavities were continuously exposed to humidified air for 30-minutes. The exposure gas for the second session consisted of a humidified air stream containing 0.36 ppm ozone. Experimental sessions were separated by at least two weeks for each subject. All subjects participated in air exposure sessions first, so that carry-over effects of the first session would be minimized.

Prior to the experimental sessions, subjects were briefly examined by the nursing staff, which was primarily checking to ensure that subjects did not have an infection. If so, the session was rescheduled. Following check-in and clearance by the nursing staff, subjects underwent a pulmonary function test (PFT) to determine baseline values of lung function parameters such as FEV1, FVC, and FEV1/FVC. This was followed by measuring baseline (pre-exposure) values for NO, Λ, and nasal airway volume (NV). Then, a unidirectional 3 l/min flow of clean air or ozonated air was administered to both nostrils while the subject quietly breathed room air through his or her mouth. Ozone uptake, NO, and NV were measured at several time points following exposure to monitor trends in these parameters. Λ and NO were measured immediately post exposure as well as 15, 30, and 60 minutes post exposure. Nasal volume was measured immediately post exposure, as well as 60 minutes post exposure. A pulmonary function

test was performed at the end of the experiment to assess changes in lung function that resulted from the experiment. A timeline of the experiment is shown in figure 3.7.

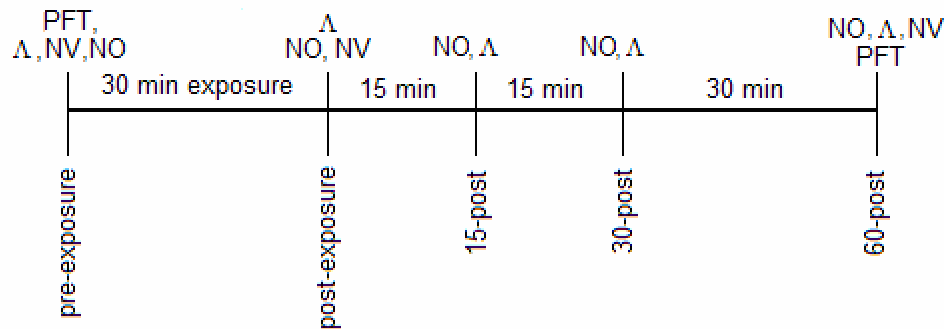


Figure 3.7. Timeline of procedures for protocol AF1

3.3.3 Protocol AF2: Determination of the influence of continuous O_3 exposure on C_{UA}, and the contribution of UA to Λ

This study investigated the response of C_{UA} as well as Λ to continuous O_3 exposure. We hypothesized that continuous exposure to O_3 would lower antioxidant levels in the NLF, namely uric acid, in a manner consistent with that of reductions in Λ. Protein levels in lavage samples were monitored to assess O_3 induced changes in epithelial permeability.

Subjects participated in two sessions, both of which were identical except for the exposure gas delivered. In the first session, subjects' nasal cavities were continuously exposed to humidified air for 30-minutes. In the second session, a humidified air stream containing 0.36 ppm O_3 served as the exposure gas. For each subject, the research sessions were separated by at least two weeks.

Following check-in by the nursing staff, subjects performed a pulmonary function test (PFT) to determine baseline lung function. This was followed by measuring baseline Λ and performing lavage. The time-point at which these baseline values were measured is referred to as '60-pre' in this study because they were measured 60 minutes prior to exposure. Sixty minutes later, Λ was measured just before initiating 30-minute nasal exposure to air or O_3. Ozone uptake was again measured immediately post-exposure followed by nasal lavage. Additional Λ measurements followed at 15, 30, and 60 minutes post exposure. Nasal lavage samples were again taken 60-minutes post-exposure immediately following the last uptake measurement. A pulmonary function test was performed at the end of the experiment to assess changes in lung function that resulted from the exposure. A timeline of the experiment is shown in figure 3.8.

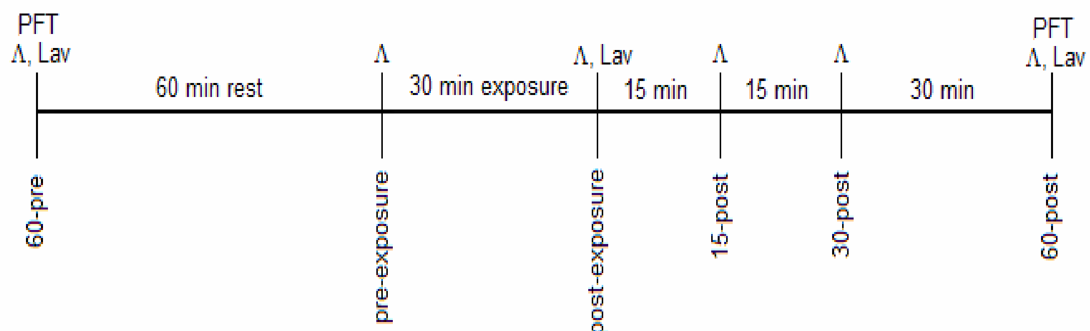


Figure 3.8 Timeline of procedures for protocol AF2

3.3.4 Protocol AF3: Effect of NO_2 exposure on Λ and C_{UA}, and determination of TBARS formation following O_3 exposure

This study was designed to investigate 1) the responses of Λ and C_{UA} to NO_2 exposure, and 2) TBARS formation following O_3 exposure. It was hypothesized that continuous exposure of the NLF to NO_2 would reduce levels of antioxidants, thus decreasing Λ. It was also hypothesized that exposure to O_3 would produce lipid peroxidation markers that could be measured by TBARS analysis of lavage samples.

Subjects participated in three sessions which were identical except for the exposure gas used. In the first session subjects' nasal cavities were continuously exposed to humidified air for 30-minutes. In the second and third sessions, a humidified air stream containing 0.36 ppm ozone or 1 ppm NO_2 served as the exposure gas. Research sessions were separated by at least two weeks for each subject.

Following check-in by the nursing staff, subjects performed a pulmonary function test (PFT) to determine baseline lung function. This was followed by measuring uptake and lavage. Sixty minutes later, Λ was measured followed by a 30-minute nasal exposure to air, O_3, or NO_2. Immediately following exposure, Λ was measured followed by lavage. Additional measurements of Λ were made 15, 30, and 60 minutes post exposure. Nasal lavage was performed 60 minutes post exposure. A pulmonary

function test was performed at the end of the experiment to assess changes in lung function that resulted from the exposure. The timeline for this protocol was the same as shown in figure 3.8.

3.4 Diffusion-Reaction Modeling

To understand the physical and chemical mechanisms of O_3 transport in the nasal cavity, we utilized a model to relate Λ to system properties, such as the reaction rate constant between O_3 and UA. In deriving the model, we assumed that ozone transport occurs laterally into the NLF from the axially convected stream. Once inside the NLF, O_3 and UA react with the rate of reactive depletion of O_3 being perfectly offset by the rate of O_3 diffusion into the NLF (steady state). A fraction of the O_3 introduced into the airways passes through the nasal cavity without reacting.

The mass balance describing lateral diffusion of O_3 in the z direction, and simultaneous reaction with uric acid in the NLF at a point along the airway surface is given as

$$D_{AB}\frac{d^2C_{O3}}{dz^2} = k_2 C_{O3} C_{UA,NLF} \tag{3.2}$$

where D_{AB} is the diffusivity of O_3 in aqueous solution, k_2 represents an assumed second order rate constant between O_3 and UA, and C_{O3} and $C_{UA,NLF}$ are the concentrations of O_3 and uric acid in the nasal lining fluid, respectively. The assumptions underlying Eq.

3.2 are that: 1) NLF is stationary; 2) axial diffusion of O_3 is negligible; 3) the concentration of UA in the NLF is unchanged during the uptake measurement, 4) there is no lateral variation of uric acid concentration throughout the NLF and 5) the system behaves in a quasi-steady manner such that the local diffusion rate is always balanced by the local reaction rate. The boundary conditions used to solve Eq. 3.2 are

$$C_{O3,\, z=0} = C_o \tag{3.3}$$

$$C_{O3,\, z=L} = 0 \tag{3.4}$$

Equation 3.3 specifies a constant liquid phase O_3 concentration at the surface of the NLF adjacent to the airway lumen, which is always in equilibrium with gas phase O_3 levels. Equation 3.4 asserts that the concentration of O_3 is diminished to zero at a finite distance, L, from the NLF surface because of rapid reaction with uric acid. Even if O_3 reaches the epithelial surface, we can assume that oxidation of cell membrane compounds occurs so rapidly that O_3 concentration reaches zero at that point. The corresponding solution for the concentration profile of O_3 is given as

$$C_{O3}(z) = C_o \frac{\sinh\left[\sqrt{k_2 C_{UA,NLF}/D_{AB}}\,(L-z)\right]}{\sinh\left[\sqrt{k_2 C_{UA,NLF}/D_{AB}}\,L\right]} \tag{3.5}$$

From this equation we identify the characteristic length for the reaction zone in the NLF as $\delta=(D_{AB}/k_2C_{UA,NLF})^{1/2}$. For the case of infinitely thick NLF, (e.g., L>>δ), the solution for the concentration profile of O_3 is given as,

$$C_{O3}(z) = C_o \exp\left[-\sqrt{\frac{k_2 C_{UA,NLF}}{D_{AB}}}\, z\right] \tag{3.6}$$

and the O_3 flux at the surface of the NLF is given by

$$N_{O3,z=0} = \sqrt{D_{AB} k_2 C_{UA,NLF}}\, C_o \tag{3.7}$$

Based on this equation, a liquid phase mass transfer coefficient can now be defined as

$$k_t \equiv \frac{N_{O3,z=0}}{C_o} = \sqrt{D_{AB} k_2 C_{UA,NLF}} \tag{3.8}$$

An overall mass transfer coefficient, K, which accounts for the combined resistance to lateral diffusion through the flowing gas and NLF phases can be determined experimentally from ozone uptake data. In particular, Aharonson et al. (1974) formulated the fractional absorption of O_3 in the nose (Λ) as,

$$\Lambda = 1 - \exp\left(-\frac{KS}{\dot{V}}\right) \tag{3.9}$$

where S and $\dot{V}$ represent the nasal surface area and volumetric flow rate of the gas stream, respectively.

The value of K can be related to individual mass transfer coefficients in the NLF (k_t) and the boundary layer formed by the flowing gas phase (k_g) by using the "resistance-in-series" concept as,

$$\frac{1}{K} = \frac{1}{k_g} + \frac{\lambda^{g,l}}{k_t} \tag{3.10}$$

where 1/K is the overall diffusion resistance, $1/k_g$ is the gas-phase resistance, $\lambda^{g,l}/k_t$ is the NLF phase resistance, and $\lambda^{g,l}$ is the equilibrium ratio of O_3 concentration at the gas-NLF interface. Santiago reported the fraction of the overall diffusion resistance due to the gas phase ($f \equiv K/k_g$) in the nasal cavities. Consequently a direct relationship can be made between K and k_t.

$$\frac{k_t}{\lambda^{g,l}} = \frac{K}{(1-f)} \tag{3.11}$$

where $f \rightarrow 0$ at large gas flow rates and $f \rightarrow 1$ as gas flow decreases.

By combining Equations 3.8, 3.9, 3.10 and 3.11 we obtain

$$-\ln(1-\Lambda) = \left[\frac{(1-f)S\sqrt{D_{AB}k_2}}{\lambda^{g,l}\dot{V}}\right] C_{UA,NLF}{}^{1/2} \qquad (3.12)$$

Recognizing further that uric acid concentrations measured in lavage samples (C_{UA}) are diluted by a factor $d \equiv (C_{UA,NLF}) / C_{UA}$, the above relationship should be written as

$$-\ln(1-\Lambda) = \left[\frac{(1-f)S\sqrt{D_{AB}k_2 d}}{\lambda^{g,l}\dot{V}}\right] (C_{UA})^{1/2} \qquad (3.13)$$

Consequently, plotting $-\ln(1-\Lambda)$ against $(C_{UA})^{1/2}$ should generate a straight line with slope equal to

$$m = \left[\frac{(1-f)S\sqrt{D_{AB}k_2 d}}{\lambda^{g,l}\dot{V}}\right] \qquad (3.14)$$

The assumed values for parameters used in this study were: $\lambda^{g,l}=6.9$, $D_{AB}=2.7\times10^{-5}$ cm^2/s, and $S=270$ cm^2 (Miller et al., 1985; Guilmette et al., 1989) Relevant values for f and d have been obtained in separate experiments (Santiago, 2001); f=0.23 at 3 lpm, f=0.08 at 10 lpm and d=11. Thus, k_2 can be computed from equation 3.14 provided that $\dot{V}$ is known.

3.5 Statistical Analysis

Statistical analysis was performed using Minitab version 13.1. Results of paired t-tests, linear regressions, analysis of variance, and analysis of covariance were considered significant at 95% confidence ($p<0.05$).

Chapter 4: Results

4.1 Results for Experimental Protocol AF1: Determination of the effect of continuous nasal O_3 exposure on subsequent Λ and NO production

Fifteen subjects were selected for this study based on criteria described in section 3.3.1.

Subject's anthropometric data are displayed in table 4.1.

Gender	Age	Height (in)	Weight (lb)
M	23	70	145
M	27	72	220
M	25	67	135
F	19	68	155
M	23	70	170
F	22	62	114
M	23	71	172
F	24	63	120
M	21	73	190
F	18	65	130
M	24	71	215
M	28	72	189
M	21	67	153
M	25	71	195
M	20	74	205
Mean	22.9	69.1	167.2
SD	2.8	3.6	34.5

Table 4.1 Anthropometric data for subjects that participated in protocol AF1

4.1.1 Influence of Continuous Ozone Exposure on Λ

Figure 4.1 shows the mean±SE trend for Λ obtained for fifteen subjects enrolled in this study during air and O_3 exposure sessions. In air sessions, paired t-tests showed no significant differences for Λ between any of the sampling times compared to pre-exposure values. In O_3 exposure sessions, Λ was significantly reduced ($p<0.001$) from a pre-exposure value of 0.825±0.019 to 0.712±0.021 immediately post-exposure. Mean Λ remained significantly reduced ($p<0.05$) 15 minutes post-exposure at 0.806. There was no significant difference between pre-exposure values and those obtained 30 minutes ($p=0.231$) and 60 minutes ($p=0.897$) post-exposure.

Individual subject trends for Λ in air and O_3 exposure sessions are displayed in figure 4.2. Each line traces Λ for each subject throughout the sessions. Figure 4.2 demonstrates a lack of consistency for subject trends in Λ during air sessions. On the other hand, the observed reduction in Λ from pre- to post-exposure measurements is pronounced in all subjects during O_3 exposure sessions. The significant depression in Λ observed 15 minutes post-exposure in O_3 exposure sessions occurred in nine of the fifteen subjects.

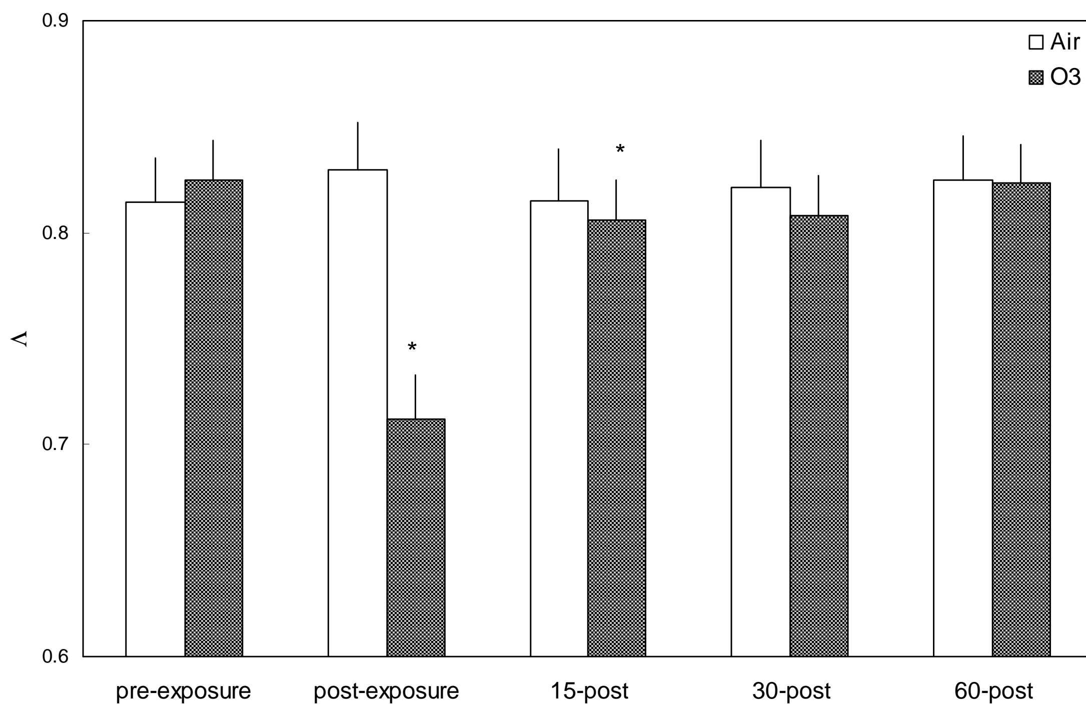


Figure 4.1. Mean±SE Λ for air and ozone sessions. Asterisks indicate values are significantly different from pre-exposure.

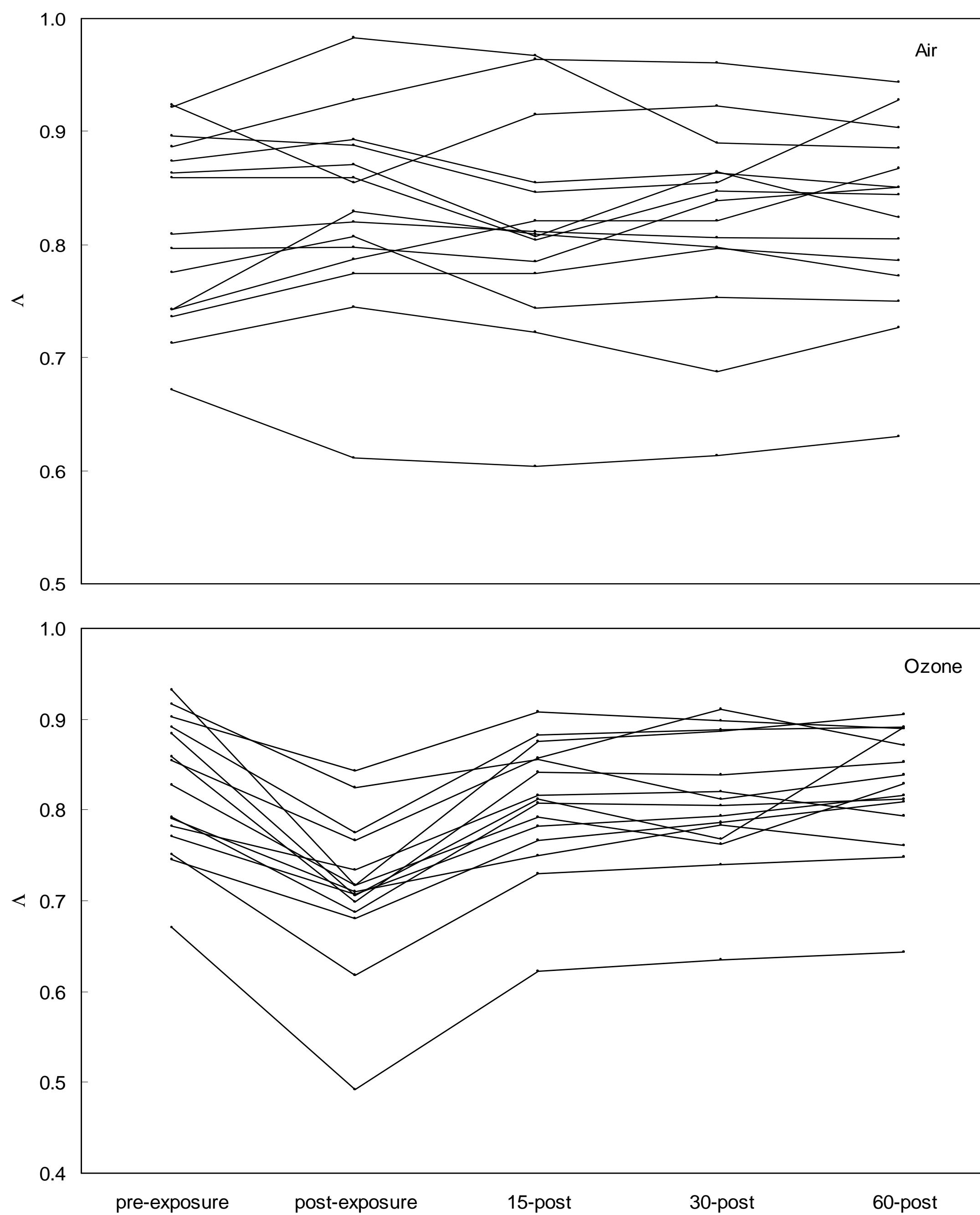
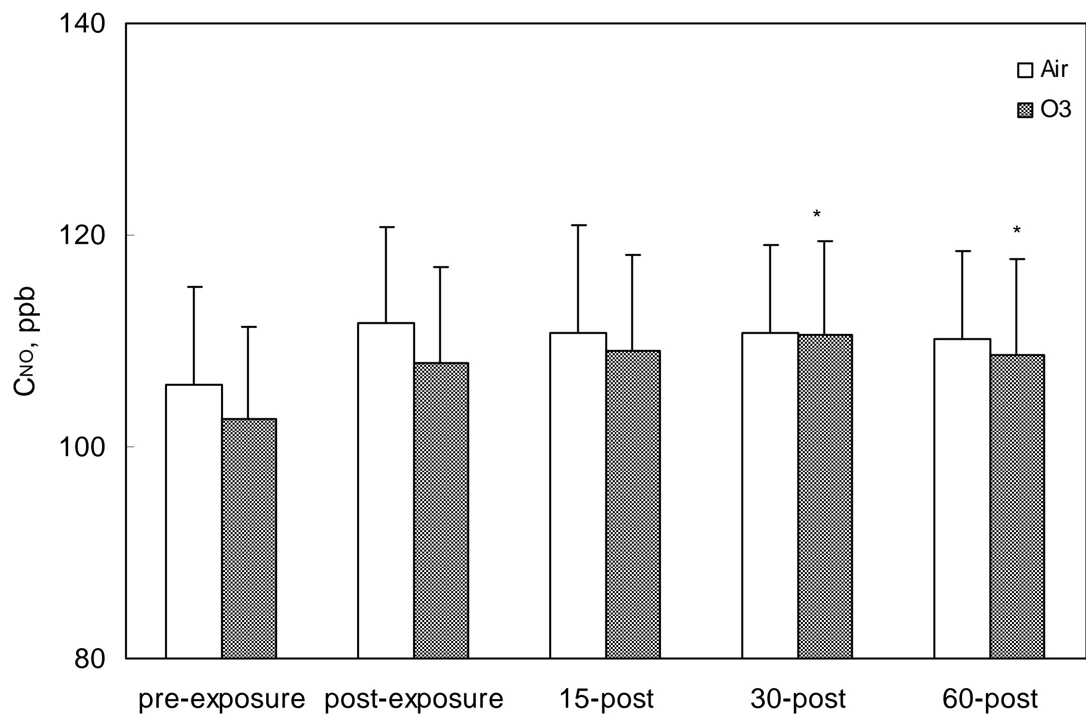


Figure 4.2 Trends in Λ for each subject during air (top) and ozone (bottom) exposure sessions. Each line traces Λ measured for one subject throughout the indicated session.

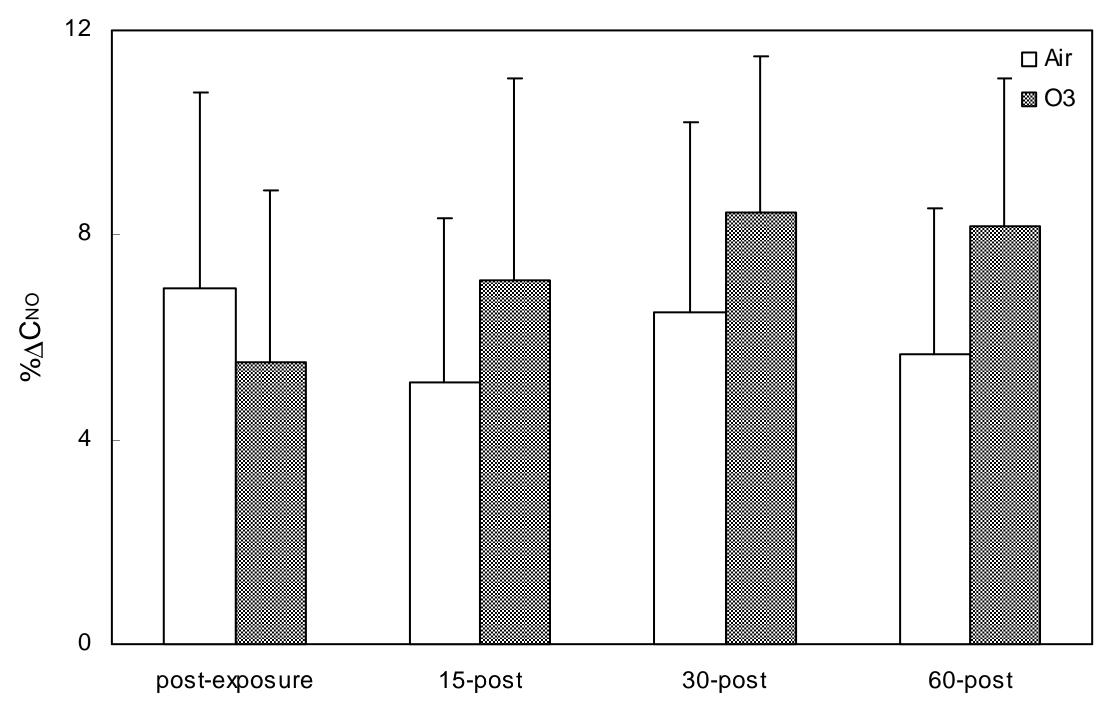
4.1.2 Response of C_{NO} to O_3 exposure

Figure 4.3 displays mean±SE C_{NO} levels for twelve subjects during air and ozone exposure sessions. Data for two subjects were removed altogether from the data pool because of difficulty in obtaining baseline C_{NO}. Data for another subject is unavailable because of NO analyzer malfunction during one session. C_{NO} was not significantly different from baseline immediately post-exposure or 15 minutes post-exposure, in O_3 exposure sessions. However, there was a small but significant elevation in C_{NO} 30 minutes ($p=0.022$) and 60 minutes ($p=0.032$) post-exposure. There was a rise in C_{NO} at all times points following air exposure; however, none were statistically different from pre-exposure values ($p\geq0.10$). To clarify C_{NO} responses following exposure, Figure 4.3 also displays percent changes in this parameter from baseline at all measurement points following exposure.

Figure 4.4 shows the individual trends for C_{NO} during air and O_3 exposure sessions. Each line traces one subject's C_{NO} levels for the indicated exposure session. In O_3 exposure sessions, nine of twelve subjects exhibited elevated NO levels 30 minutes post-exposure, and eight of eleven subjects exhibited this trend 60-minutes post-exposure. This was true for eight of thirteen and eleven of thirteen subjects at 30 and 60 minutes post air exposure, respectively. Despite the common C_{NO} trend following both exposures, nine of twelve and eight of eleven subjects exhibited greater elevations in C_{NO} at 30 and 60 minutes post-exposure in O_3 sessions than in air sessions. Figure 4.5 directly compares pre-exposure to 30-minute and 60-minute post-exposure values of C_{NO} for each subject.



140
120
100
80
CNO, ppb
Air
O3
*
*
pre-exposure
post-exposure
15-post
30-post
60-post



12
8
4
0
%ΔCNO
Air
O3
post-exposure
15-post
30-post
60-post

Figure 4.3. Top: Mean±SE C_{NO} levels for air and O_3 exposure sessions. Asterisks indicate values are significantly different from pre-exposure. Bottom: Mean±SE percent changes in C_{NO} at sampling points following exposure.

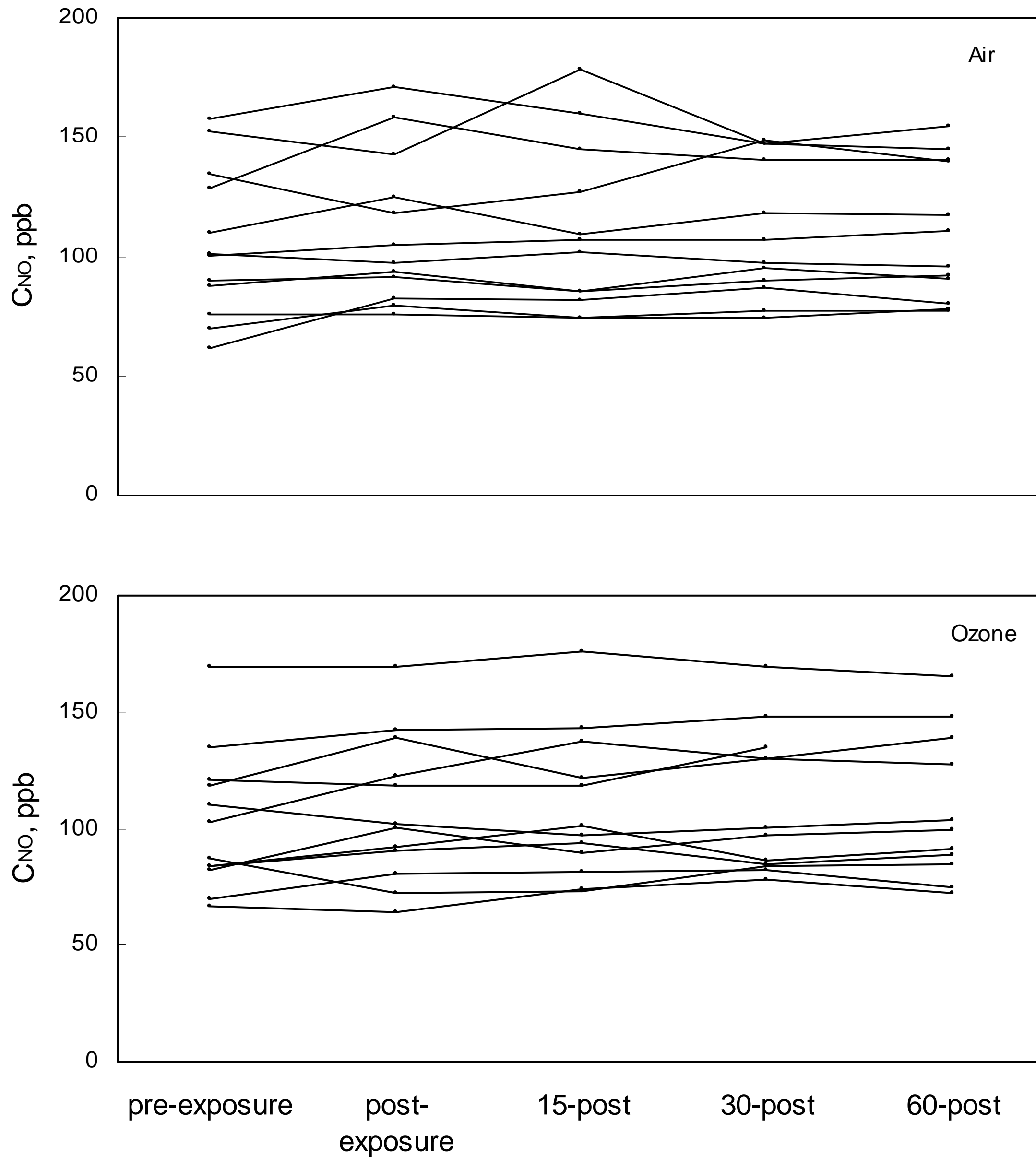


Figure 4.4 Nitric oxide trends for individual subjects during air (top) and ozone (bottom) exposure sessions. Each line follows C_{NO} for one subject throughout the indicated session.

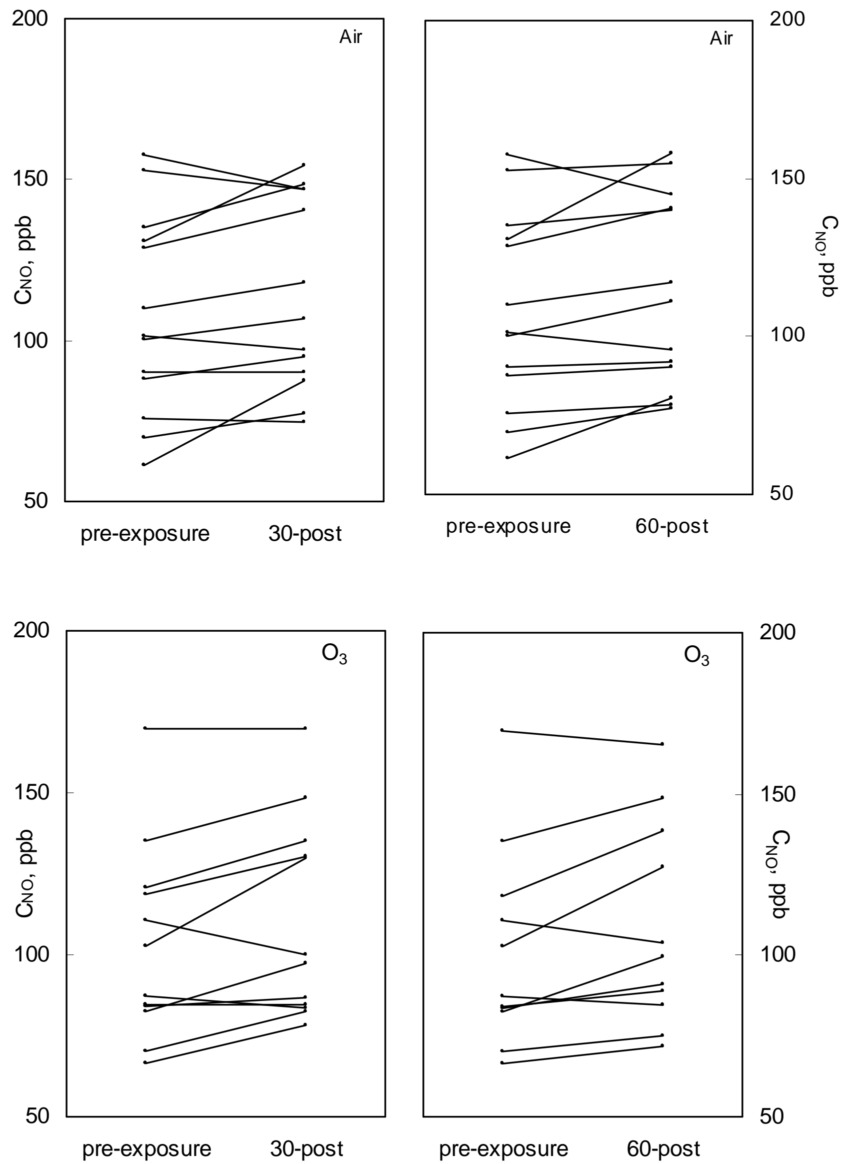


Figure 4.5 Direct comparison of C_{NO} for each subject between pre-exposure and post exposure measurements for air and O_3 exposure sessions: 30 minute post exposure (left panels), 60 minute post exposure (right panels).

4.1.3 Changes in NV following exposure

Figure 4.6 displays mean±SE nasal volume for the subjects that were enrolled in this study. Paired t-tests indicated that there was a significant reduction in nasal volume for air sessions immediately post exposure ($p=0.018$) but not 60 minutes post-exposure ($p=0.227$). Mean NV was reduced in a similar fashion for O_3 exposure sessions. However, a significant change from baseline did not occur at any measurement points for O_3 exposure sessions, as the trend was inconsistent among the subject's data.

Figure 4.7 shows individual subject trends for nasal volume during air and ozone exposure sessions. In air exposure sessions, eleven out of fourteen subjects displayed a reduction in nasal volume immediately post-exposure. This occurred for nine out of thirteen subjects in O_3 exposure sessions.

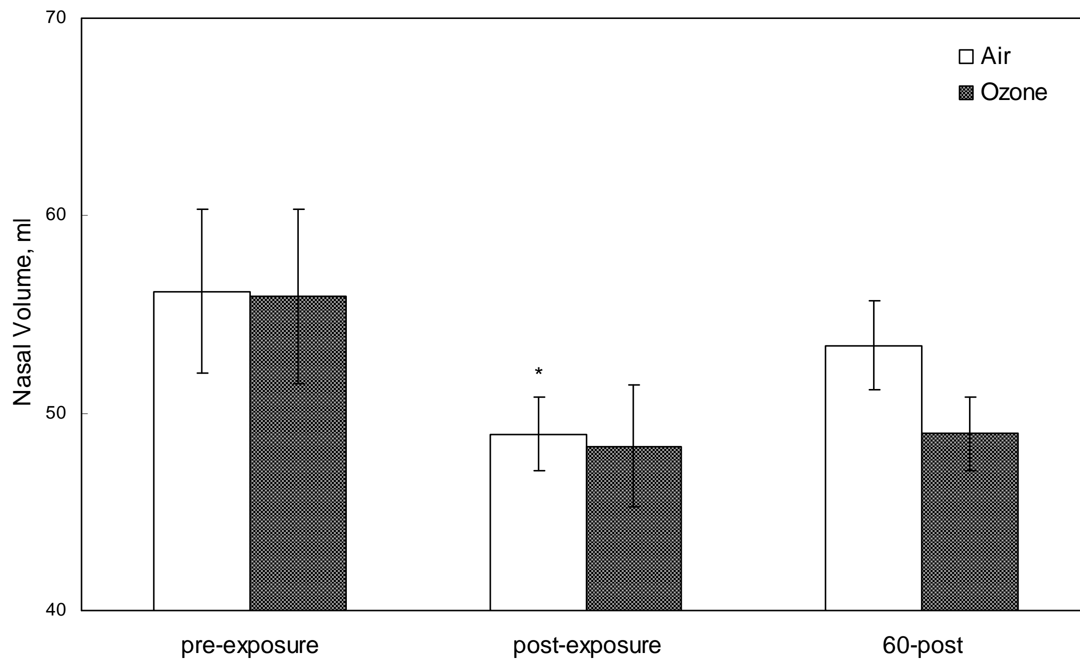


Figure 4.6. Mean±SE nasal volume for air and ozone sessions. Asterisks indicate values are significantly different from pre-exposure.

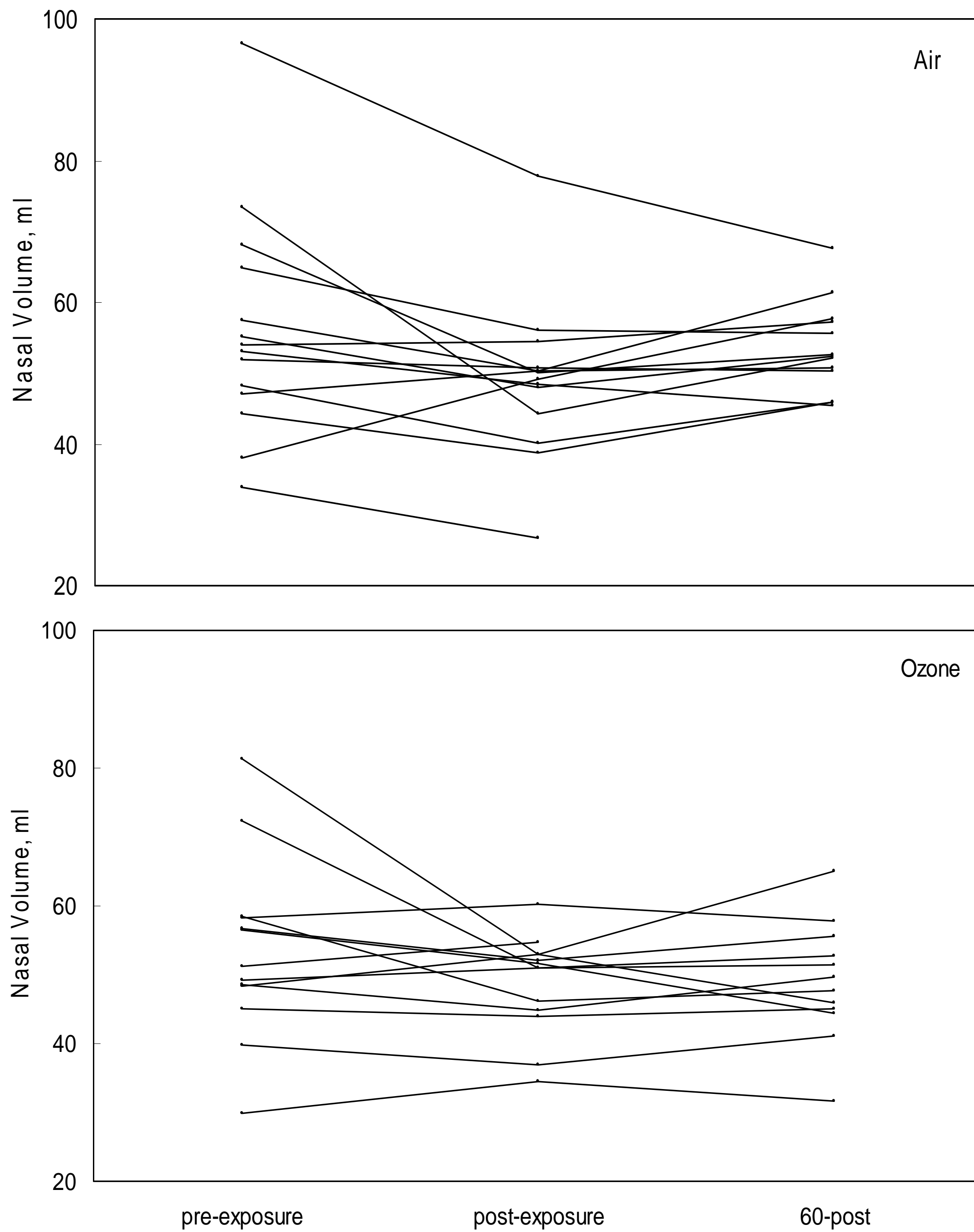


Figure 4.7 Trends in nasal volume for individual subjects during air (top) and O_3 (bottom) exposure sessions. Each line represents nasal volume for one subject throughout the indicated session.

4.2 Results for Experimental Protocol 4.2 AF2: Determination of the influence of continuous O_3 exposure on C_{UA}, and the contribution of UA to Λ

Twenty-five subjects were enrolled in this study. Subjects were recruited and screened based on criteria described in Chapter 3. The subject's anthropometric data are displayed in Table 4.2.

gender	Age	height (in)	weight (lbs)
M	25	71	210
F	24	62	160
F	24	67	125
F	19	65	125
M	25	67	180
M	32	69	180
M	20	70	220
F	19	66	140
M	19	69	155
M	20	71	158
F	21	63	121
M	27	71	220
F	25	65	175
F	22	66	125
F	22	64	125
F	19	63	210
M	23	72	203
F	21	66	140
M	19	67	135
M	19	68	150
M	20	67	135
F	19	63	135
M	19	68	170
M	31	72	185
F	20	63	125
Mean	22.2	67.0	160.3
SD	3.7	3.1	33.1

Table 4.2 Anthropometric data for subjects participating in protocol AF2.

4.2.1 Influence of Continuous Ozone Exposure on Λ

Figure 4.8 displays mean±SE Λ for air and O_3 exposure sessions. The experimental protocol in this section differs from that in section 4.1.1 for two reasons: 1) lavage was performed at several time points in this study, whereas AF1 did not include lavage and 2) this study included an added pre-exposure measurement 60-minutes prior to exposure.

There was a small, increase in mean Λ from 60-minutes pre-exposure to immediately pre-exposure that was insignificant during the air sessions ($p=0.063$) and O_3 sessions ($p=0.054$). A paired t-test comparison between Λ measured immediately pre-air exposure and 15-minutes post-air exposure indicated a significant reduction ($p=0.027$), which was consistent in eighteen of twenty-five subjects. No significant results arose from paired t-test comparisons between Λ measured 60-minutes pre-exposure and at any time point following air exposure.

In O_3 exposure sessions, paired t-tests suggested that Λ was significantly reduced from 60-minutes pre-exposure to immediately post-exposure ($p<0.001$). This reduction was consistent in all but one subject. Similarly, compared to Λ immediately pre-exposure, all subjects underwent a significant reduction immediately post exposure ($p<0.001$). Compared to immediately pre-exposure, mean Λ remained significantly depressed 15-

minutes post exposure ($p<0.01$). This was observed for sixteen of twenty-four subjects. Individual subject trends for air and O_3 exposure sessions are shown in figure 4.9.

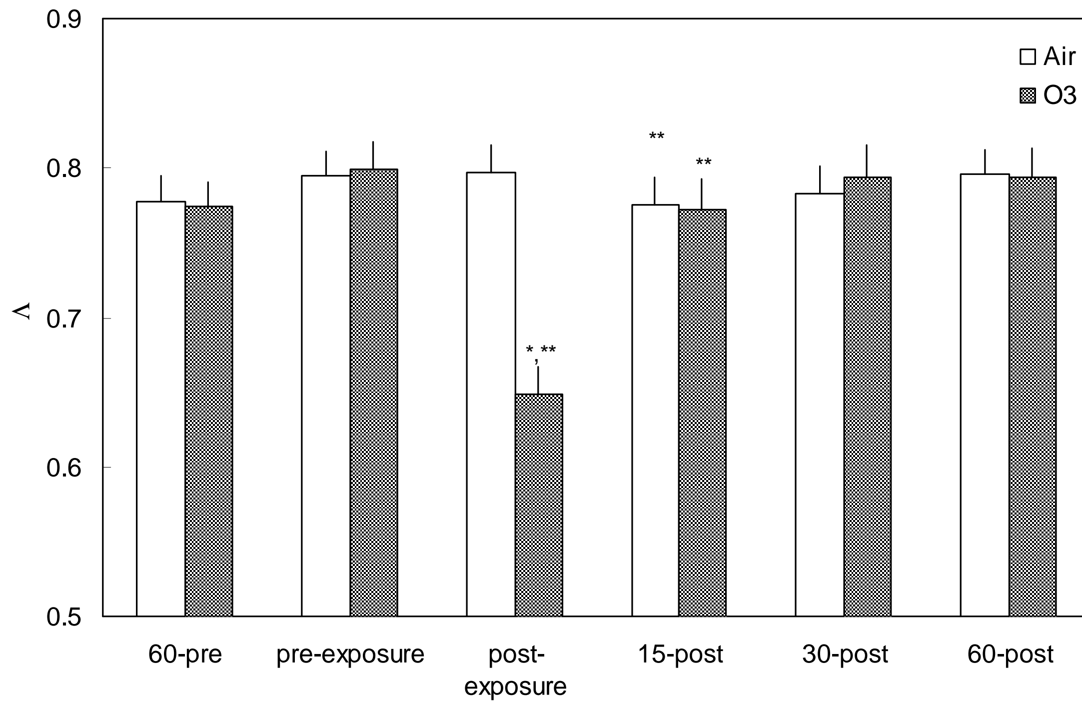


Figure 4.8. Mean±SE Λ for air and ozone exposure sessions. * indicates mean is significantly different from 60-pre. ** indicates mean is significantly different from pre-exposure.

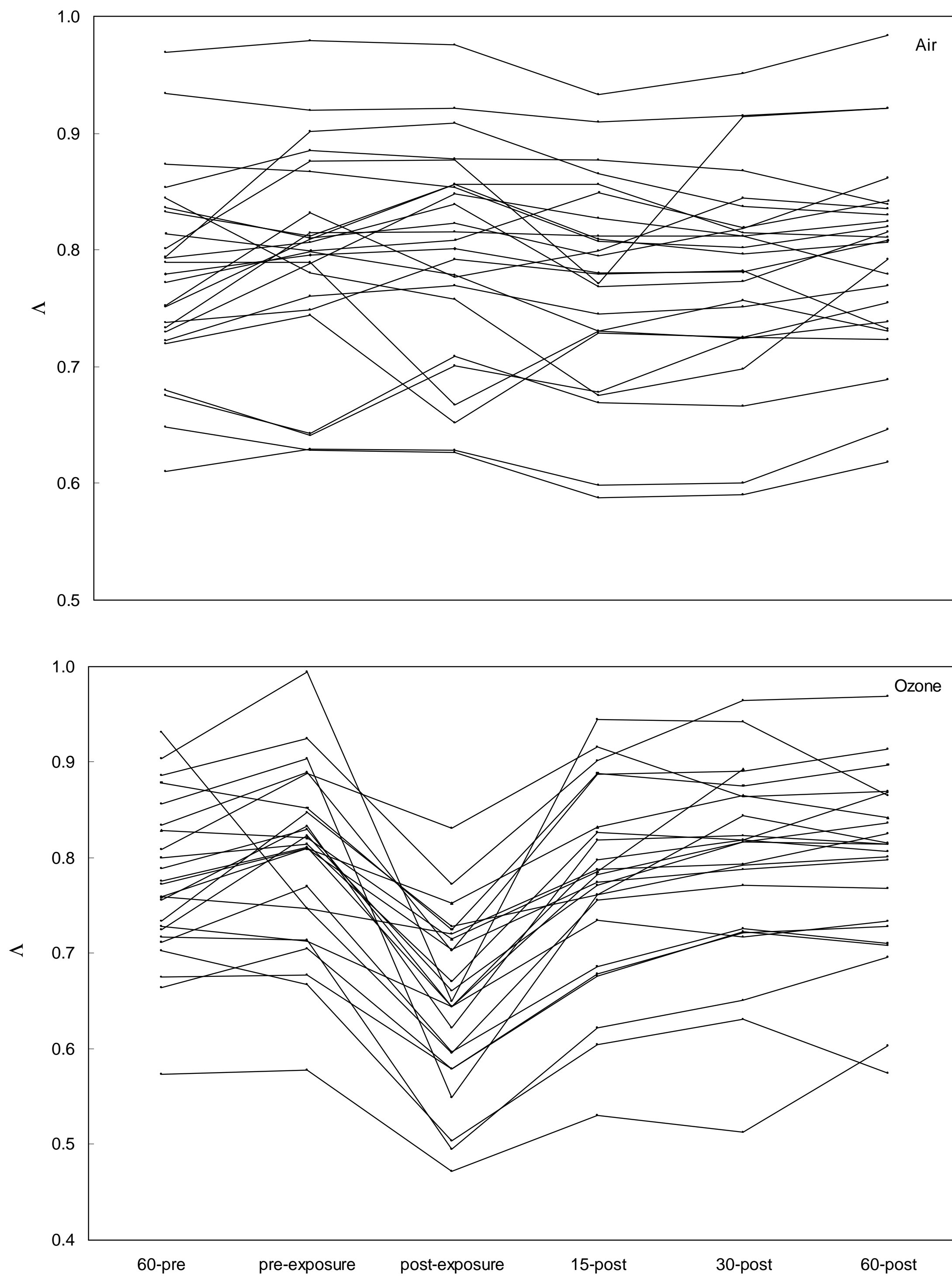


Figure 4.9 Trends in Λ for each subject during air (top) and ozone (bottom) exposure sessions. Each line traces Λ for one subject throughout the indicated session.

4.2.2 Effect of Continuous Ozone Exposure on C_{UA}

Figure 4.10 shows mean±SE C_{UA} values for the subjects enrolled in this study during air and O_3 exposure sessions. One subject was removed from C_{UA} analysis in air sessions because pre-exposure C_{UA} levels were five standard deviations greater than the mean. One subject participated in the air exposure session, but withdrew prior to the O_3 exposure session. Data obtained in air sessions for this subject were included in the final analysis.

Results of paired t-tests indicated that mean C_{UA} was significantly increased ($p=0.028$) in air sessions from 60-pre-exposure compared to immediately-post-exposure measurements. There was no significant difference between 60-pre and 60-post values of C_{UA} during air exposure sessions ($p=0.84$). There was a significant reduction ($p<0.001$) in C_{UA} during O_3 exposure sessions between 60-pre and immediate post-exposure measurements. There was no significant difference ($p=0.284$) in C_{UA} between 60-pre and 60-post values of C_{UA} during O_3 exposure sessions. Individual subject trends in C_{UA} for the two sessions are provided in figure 4.11.

Figure 4.11 shows that in air sessions, seventeen of twenty-four subjects displayed augmented C_{UA} immediately post-exposure compared to baseline. On the other hand,

twenty-one out of twenty-four subjects exhibited a reduction in C_{UA} immediately post-exposure in O_3 exposure sessions.

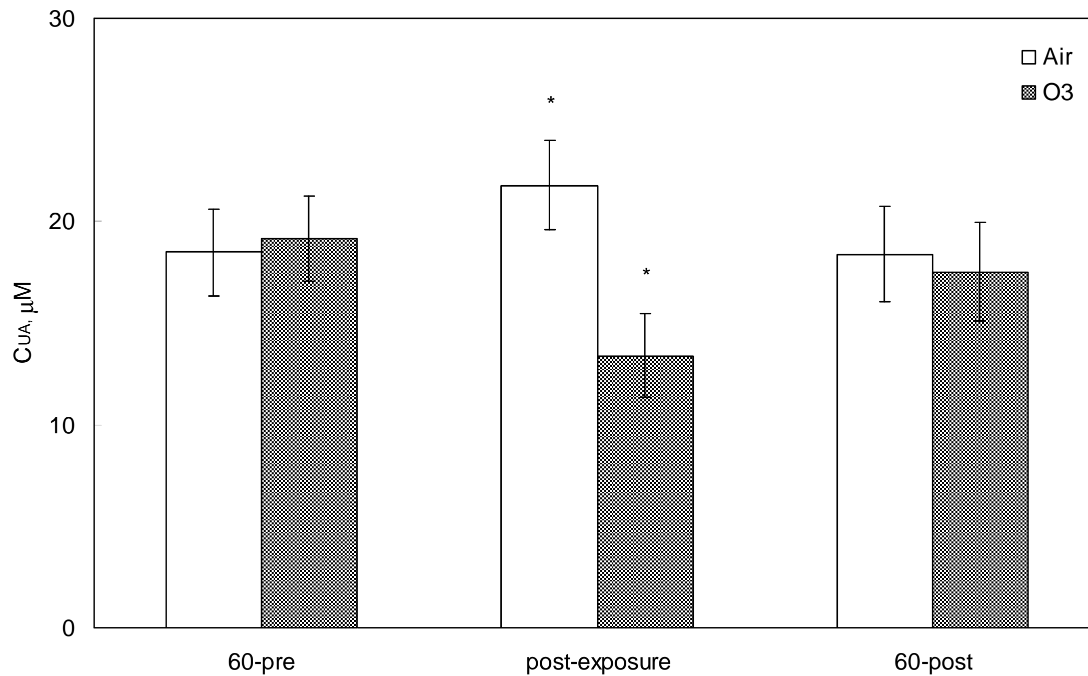


Figure 4.10 Mean±SE C_{UA} values for air and ozone sessions. Asterisks indicate values are significantly different from 60-pre.

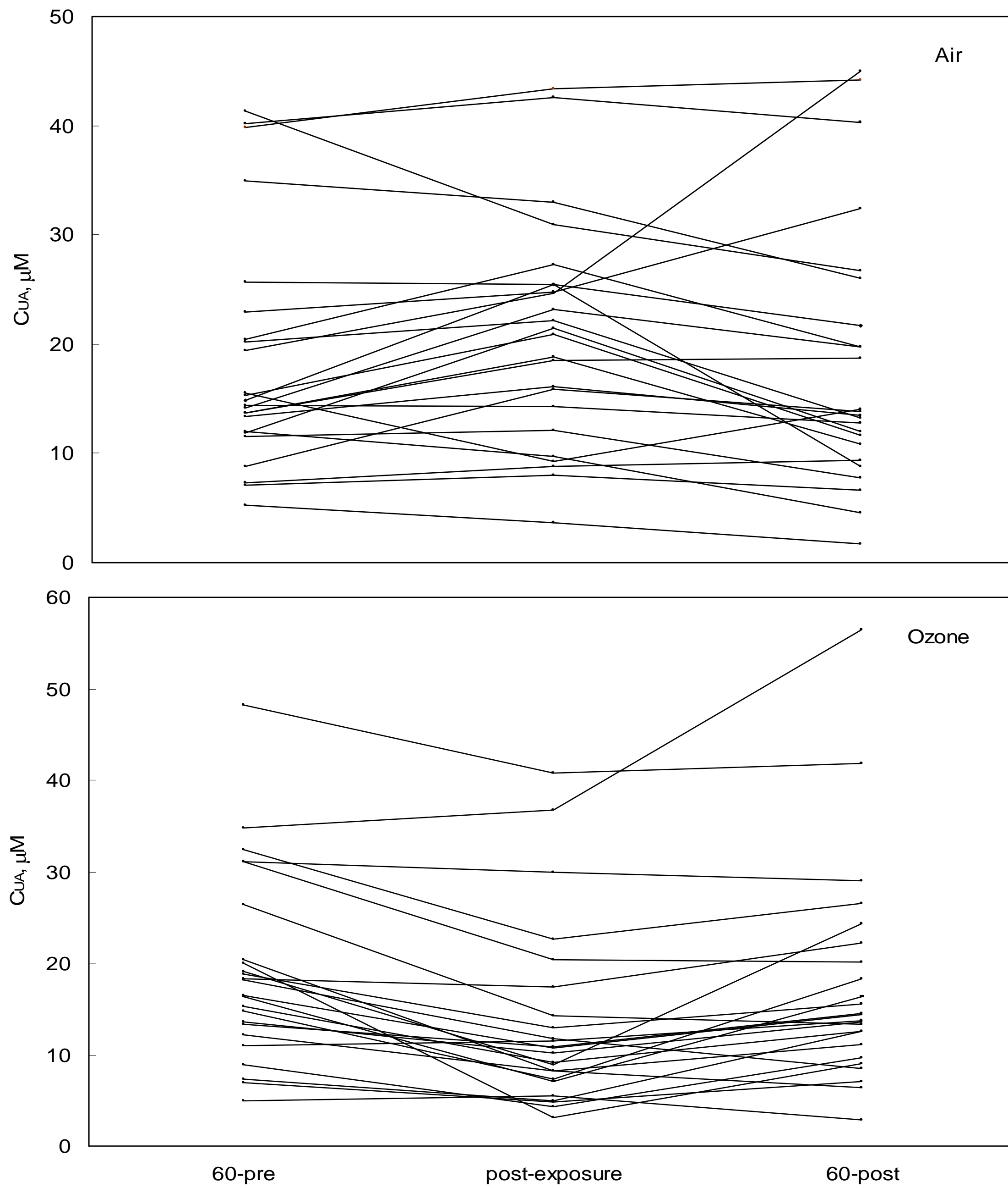


Figure 4.11 Trends in C_{UA} for each subject during air (top) and O_3 (bottom) exposure sessions. Each line represents C_{UA} measured for one subject throughout the indicated session.

4.2.3 Effect of Continuous Ozone Exposure on protein levels

Figure 4.12 shows the mean±SE trend in protein levels for twenty-one subjects. Three subject's data were not considered in the analysis because their protein levels were greater than three standard deviations from the mean value for at least one data point. One subject participated in the air exposure session, but withdrew from the study prior to O_3 exposure. Data for this subject was not included in the final analysis.

Paired t-tests did not suggest significant differences between protein levels measured at any time points during air exposure sessions ($p>0.4$). In O_3 exposure sessions, protein levels were not significantly different from baseline in samples taken immediately post exposure ($p=0.72$). Compared to baseline, there was an insignificant reduction in protein 60-minutes post-exposure ($p=0.051$).

Trends in protein levels for each subject are shown in figure 4.13. The significant reduction 60-minutes post O_3 exposure occurred in thirteen out of the twenty-one subjects.

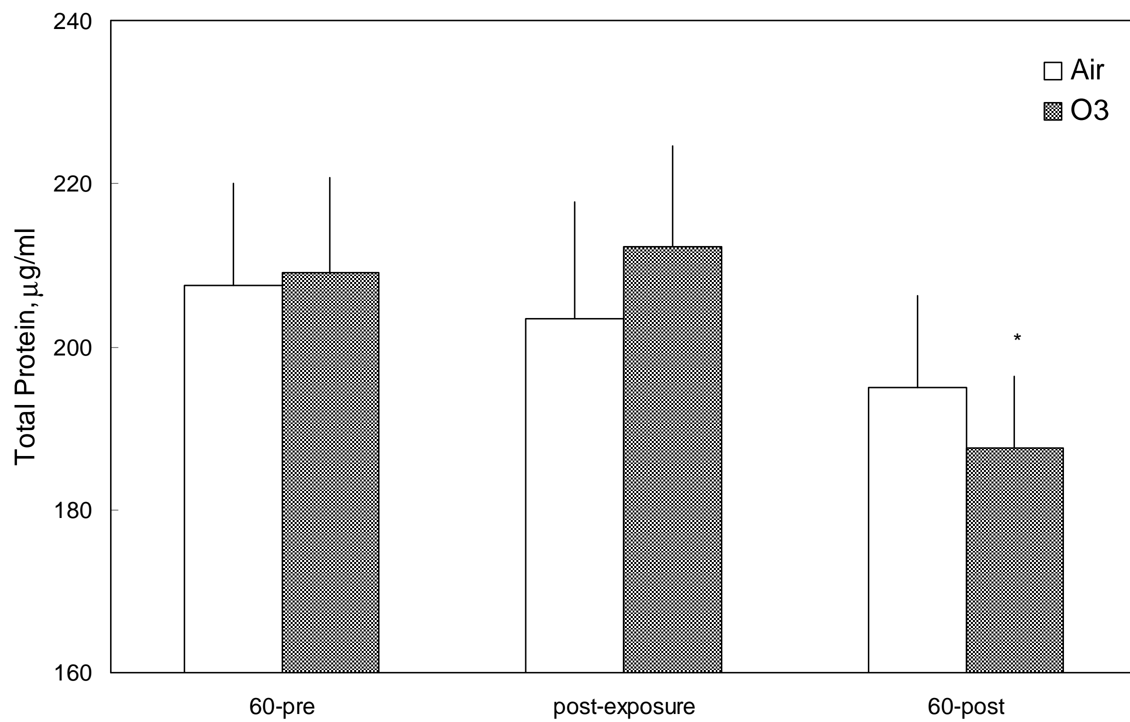


Figure 4.12. Mean Total Protein±SE for air and ozone sessions. Asterisks indicate values are significantly different from pre-exposure.

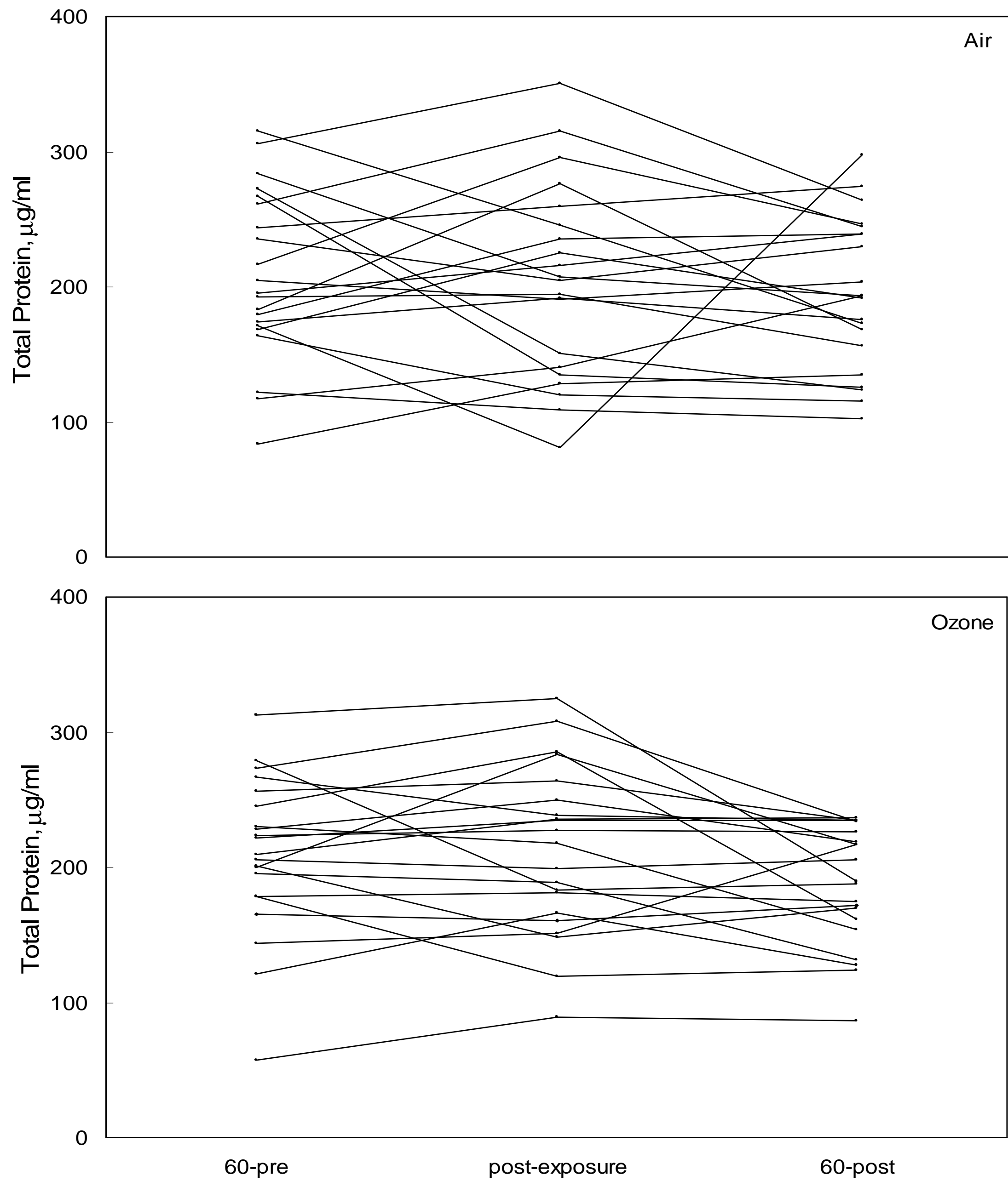


Figure 4.13 Total Protein trends for each subject during air (top) and O_3 (bottom) exposure sessions. Each line indicates the trend in total protein throughout the indicated session

4.2.4 Reaction-Diffusion Modeling

Correlations resulting from day-to-day variations in Λ and C_{UA}

Figure 4.14 relates Λ and C_{UA} for each subject on two different days. Each line segment connects two baseline measurements of Λ and C_{UA} taken for each subject on the two separate days they came to the lab. One subject was excluded from analysis in this section because they exhibited greater than a five fold change in C_{UA} between the two days.

By applying equation 3.13 to the data in figure 4.14, we generated a plot of $-\ln(1-\Lambda)$ against $C_{UA}^{1/2}$, seen in figure 4.15. An ANCOVA performed on this data, with $-\ln(1-\Lambda)$ as the predicted variable, subject as a random factor, and $C_{UA}^{1/2}$ as a covariate, yields an insignificant correlation ($p=0.185$).

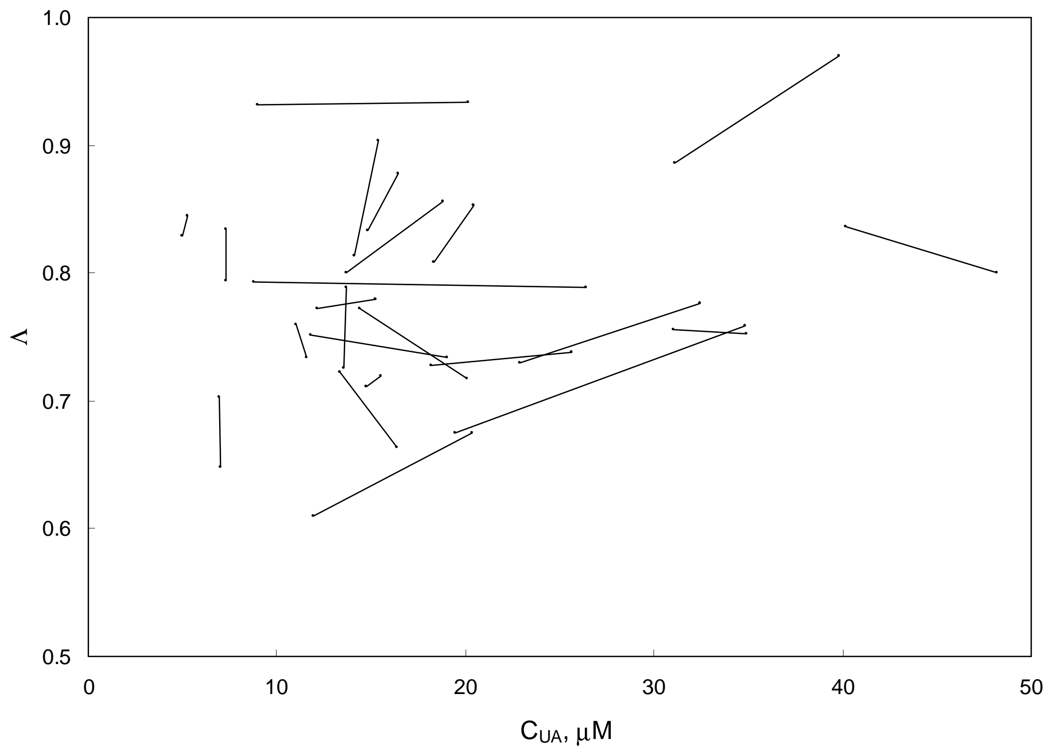


Figure 4.14 Relationship between Λ and C_{UA} measured at baseline for each subject on the two experimental days. Each line connects two data points obtained for one subject on the two days.

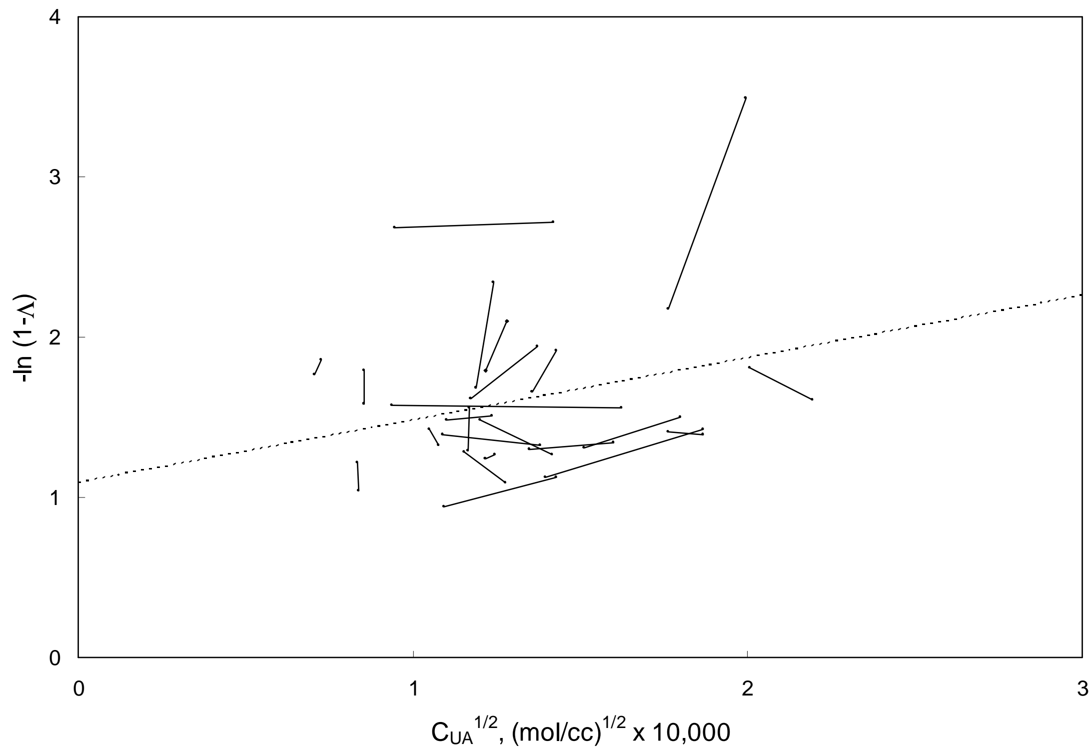


Figure 4.15 Relationship between baseline values for $-\ln(1-\Lambda)$ and $C_{UA}^{1/2}$ for each subject on the two experimental days. Each line connects data obtained for one subject on the two days. The dashed line represents the regression for this data obtained from ANCOVA. The slope of this regression was insignificant (p=0.185).

Correlations resulting from variations in Λ and C_{UA} resulting from air exposure

Figure 4.16 displays a crossplot of Λ and C_{UA} for each subject before and after exposure to clean air. Each line segment connects two data points for Λ and C_{UA} acquired for each subject sixty minutes pre-exposure and immediately post-exposure to air.

Conversion of this data using eq 3.13 and plotting $-\ln(1-\Lambda)$ against $C_{UA}^{1/2}$ yields figure 4.17. An ANCOVA performed on this data, with $-\ln(1-\Lambda)$ as the predicted variable, subject as a random factor, and $C_{UA}^{1/2}$ as a covariate, failed to show a significant correlation ($p=0.076$).

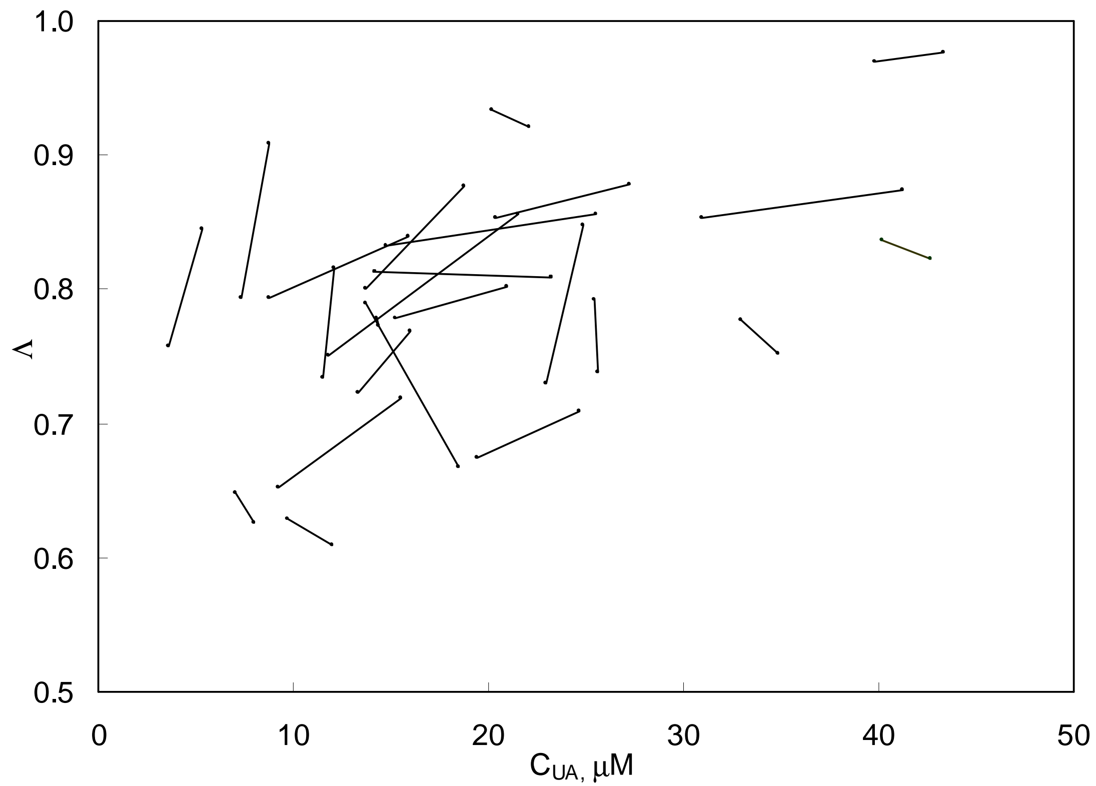


Figure 4.16 Relationship between Λ and C_{UA} measured at baseline and immediately post-exposure for each subject during air exposure sessions. Each line connects two data points obtained for one subject at the 60-pre, and post-exposure measurements.

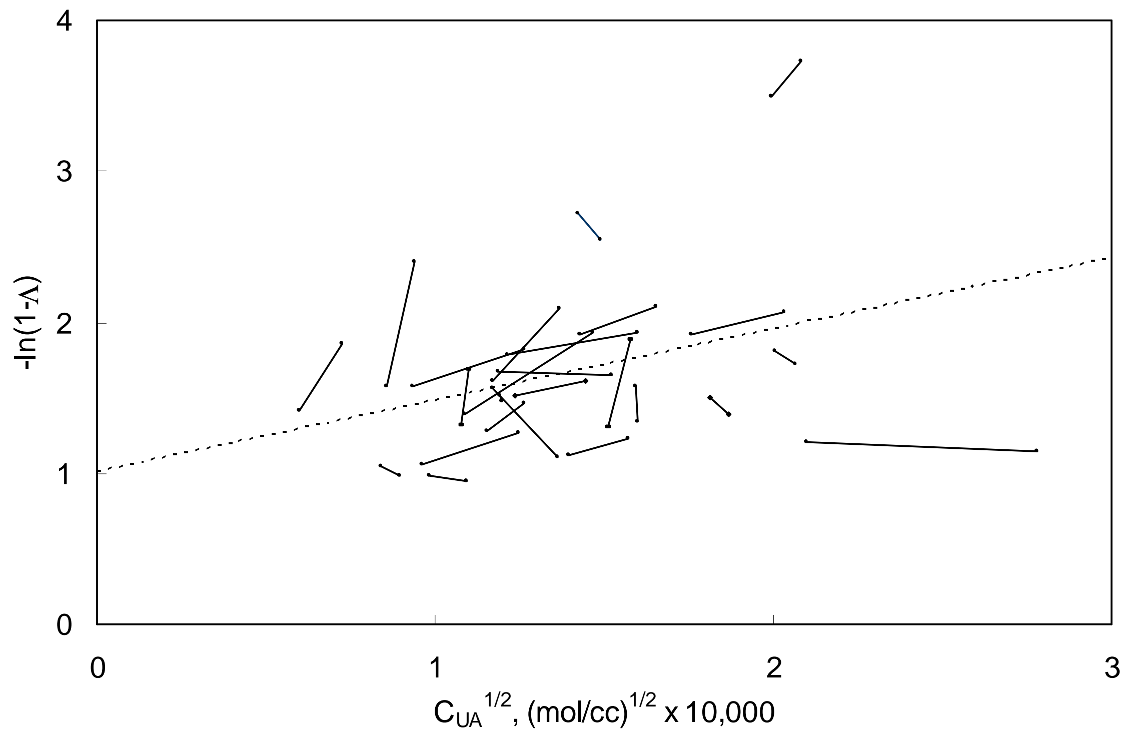


Figure 4.17 Relationship between –ln(1-Λ)and $C_{UA}^{1/2}$ for each subject before and after air exposure. Each line connects data obtained for one subject at the 60-pre and post-exposure measurements. The dashed line represents the regression for this data obtained from ANCOVA. The slope of this regression was insignificant (p=0.076).

Correlations resulting from variations in Λ and C_{UA} resulting from ozone exposure

Figure 4.18 displays Λ and C_{UA} for each subject before and after exposure to 0.36 ppm O_3. Each line segment connects two data points for Λ and C_{UA} collected for each subject sixty minutes pre-exposure and immediately post-exposure to O_3.

Application of equation 3.13 to the data in figure 4.18 yielded a plot of $-\ln(1-\Lambda)$ against $C_{UA}^{1/2}$ shown in figure 4.19. An ANCOVA performed on this data with $-\ln(1-\Lambda)$ as the predicted variable, subject as a random factor, and $C_{UA}^{1/2}$ as a covariate yields a significant correlation ($p<0.001$) with slope of 13376±3181 $(mol/cc)^{-1/2}$. The value for the intercept of the regression was -0.306±0.390, and was not significantly different from zero ($p=0.441$). According to equation 3.14, this slope corresponds to an apparent k_2 value (mean±SE) of $1.56 \times 10^9 \pm 0.74 \times 10^9$ $M^{-1}s^{-1}$.

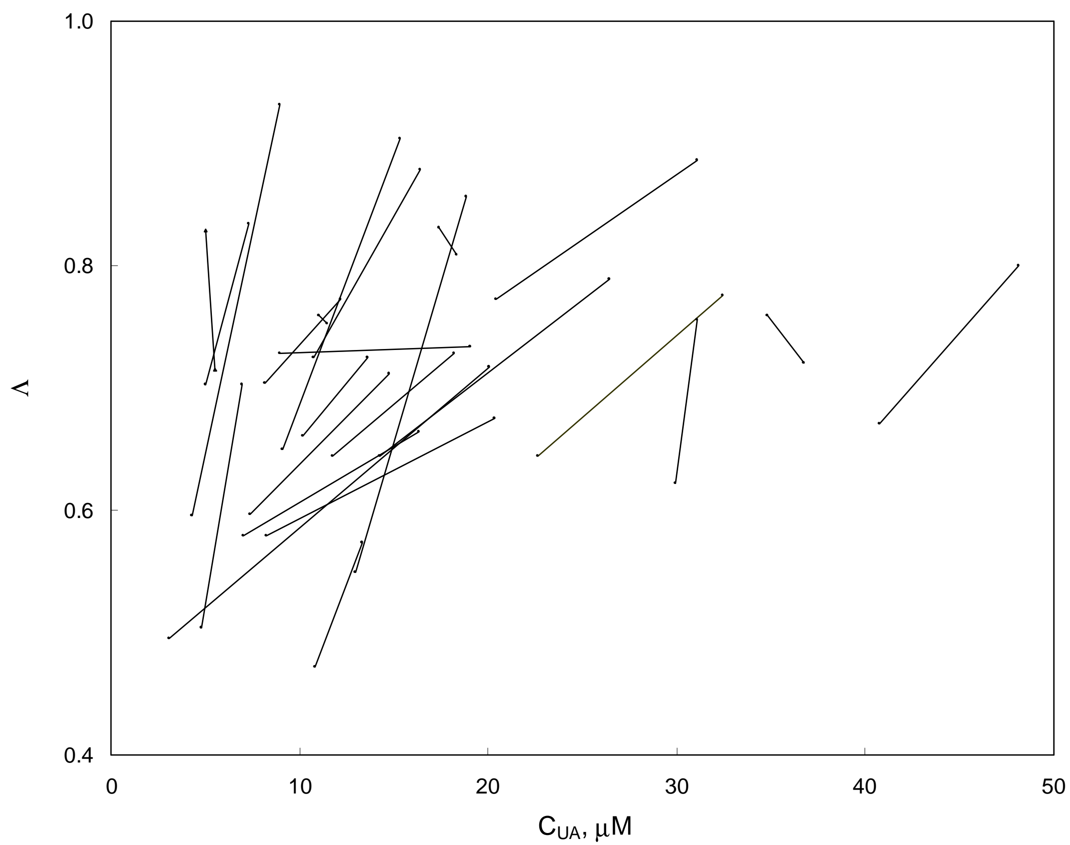


Figure 4.18 Relationship between Λ and C_{UA} measured at baseline and immediately post-exposure for each subject in O_3 exposure sessions. Each line connects two data points obtained for one subject at the 60-pre, and post-exposure sampling points.

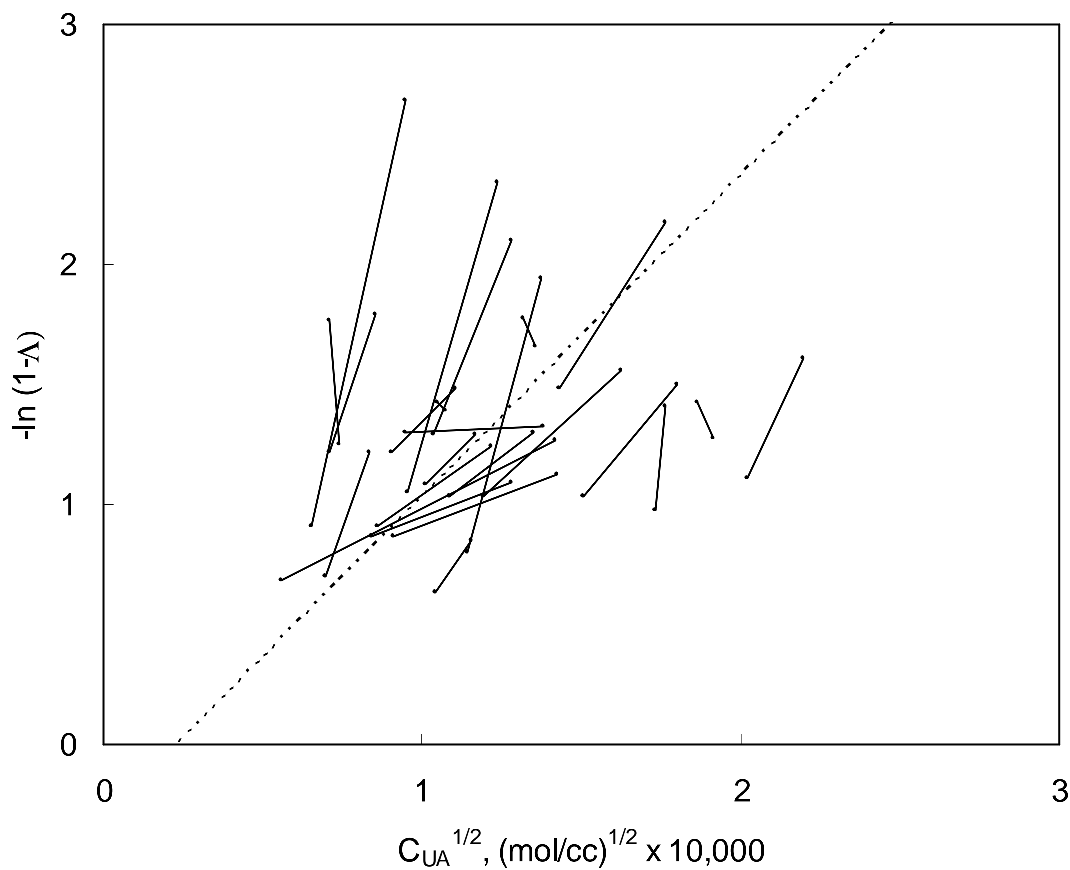


Figure 4.19 Relationship between $-\ln(1-\Lambda)$ and $C_{UA}^{1/2}$ for each subject before and after O_3 exposure. Each line connects data obtained for one subject at the 60-pre and post-exposure measurements. The dashed line represents the regression for this data obtained from ANCOVA. The slope of this regression was significant ($p<0.001$) at a value of 13376±3181 $(cc/mol)^{1/2}$.

4.3 Results for Protocol AF3: Effect of NO_2 exposure on Λ and C_{UA}, and the effect of O_3 exposure on TBARS formation

Twelve subjects completed this study. Anthropometric data for these subjects are given in table 4.3. It must be noted that the subjects who participated in NO_2 exposure sessions were a subset of those included in the AF2 study. Therefore, for direct comparison of effects of separate air, O_3, and NO_2 exposures, the results from study AF3 have been co-presented with those of AF2.

Gender	Age	Height (cm)	Weight (lb)
M	27	71	220
F	25	65	175
F	22	66	125
F	22	64	125
F	19	63	210
M	23	72	203
F	21	66	140
M	19	67	135
M	19	68	150
M	20	67	135
F	19	63	135
M	19	68	170
M	31	72	185
F	20	63	125
Mean	21.9	66.8	159.5
SD	3.6	3.2	33.9

Table 4.3 Anthropometric data for subjects participating in protocol AF3.

4.3.1 Influence of Continuous NO_2 Exposure on Λ

Figure 4.20 displays mean±SE Λ for twelve subjects that participated in NO_2 exposure sessions. There was an insignificant ($p=0.09$) increase in Λ 60-minutes after baseline, indicated by a paired t-test. Also compared to baseline, there was no significant difference in Λ observed immediately post NO_2 exposure ($p=0.889$). However, compared to Λ measured immediately pre-exposure, ten of twelve subjects underwent a reduction immediately post exposure. A paired t-test indicated that this reduction was significant at $p=0.049$. Λ remained depressed for nine subjects 15 minutes post-exposure ($p=0.039$). Trends in Λ throughout NO_2 exposure sessions are presented in figure 4.21 for each subject.

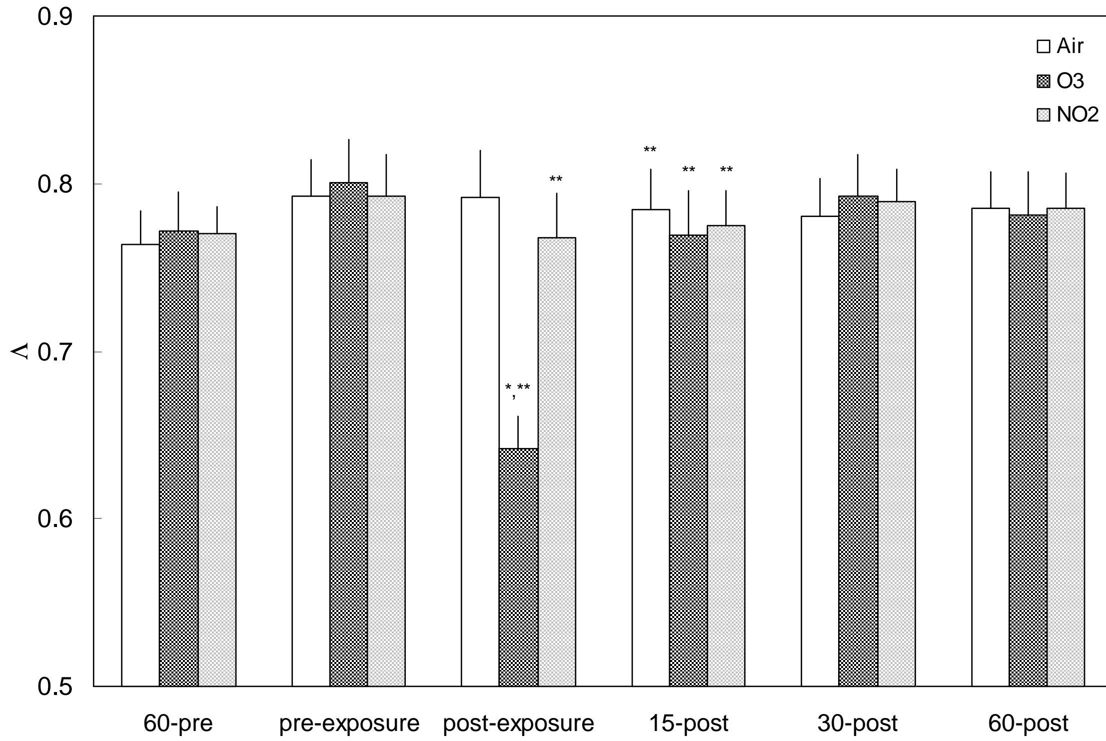


Figure 4.20. Mean±SE Λ for air, O_3 and NO_2 exposure sessions for the twelve subjects that participated in protocol AF3. * indicates mean is significantly different from 60-pre. ** indicates mean is significantly different from immediately pre-exposure.

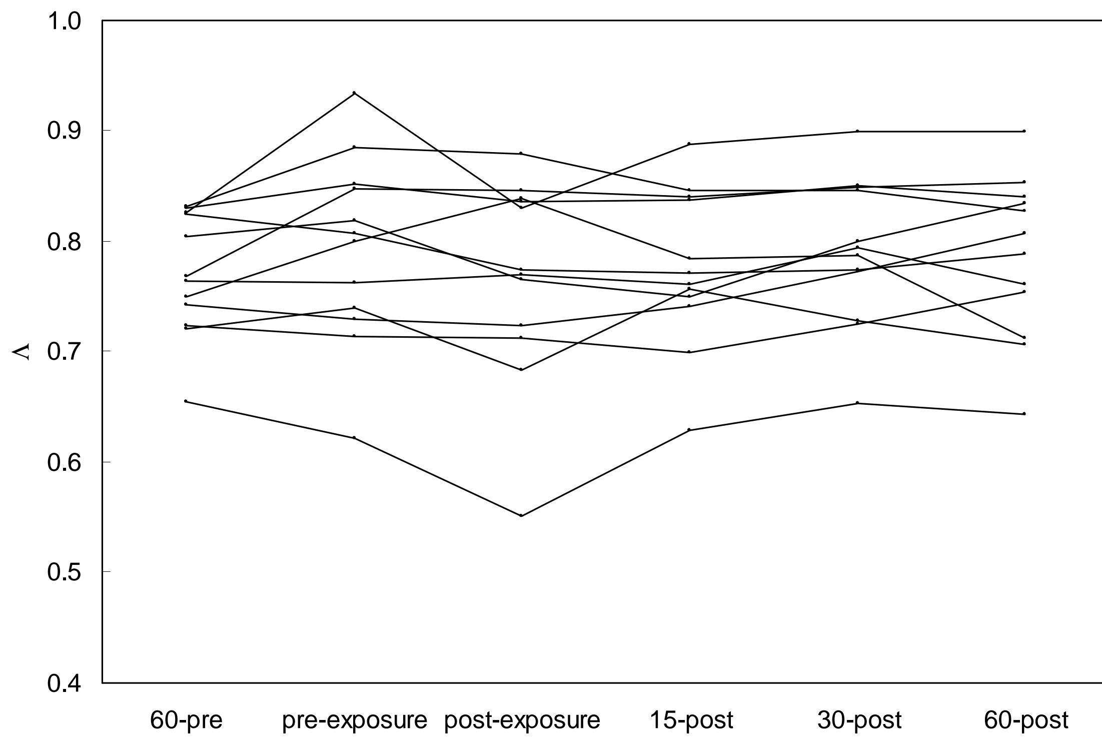


Figure 4.21. Trends in Λ for each subject throughout NO_2 exposure sessions. Each line represents Λ for one subject measured throughout the session.

4.3.2 Influence of Continuous NO_2 Exposure on C_{UA}

Figure 4.22 shows mean±SE C_{UA} values for the subjects enrolled in this study for NO_2 exposure sessions. One subject participated in the air exposure session, but withdrew prior to O_3 and NO_2 exposure sessions. Another subject withdrew because they began taking prescription medications after participating in air and O_3 sessions. Data obtained in air sessions for these two subjects were not considered in the final analysis.

Results of paired t-tests fail to suggest significant changes in C_{UA} from baseline throughout NO_2 exposure sessions ($p>0.2$). Figure 4.23 traces individual trends in C_{UA} for each subject in this study.

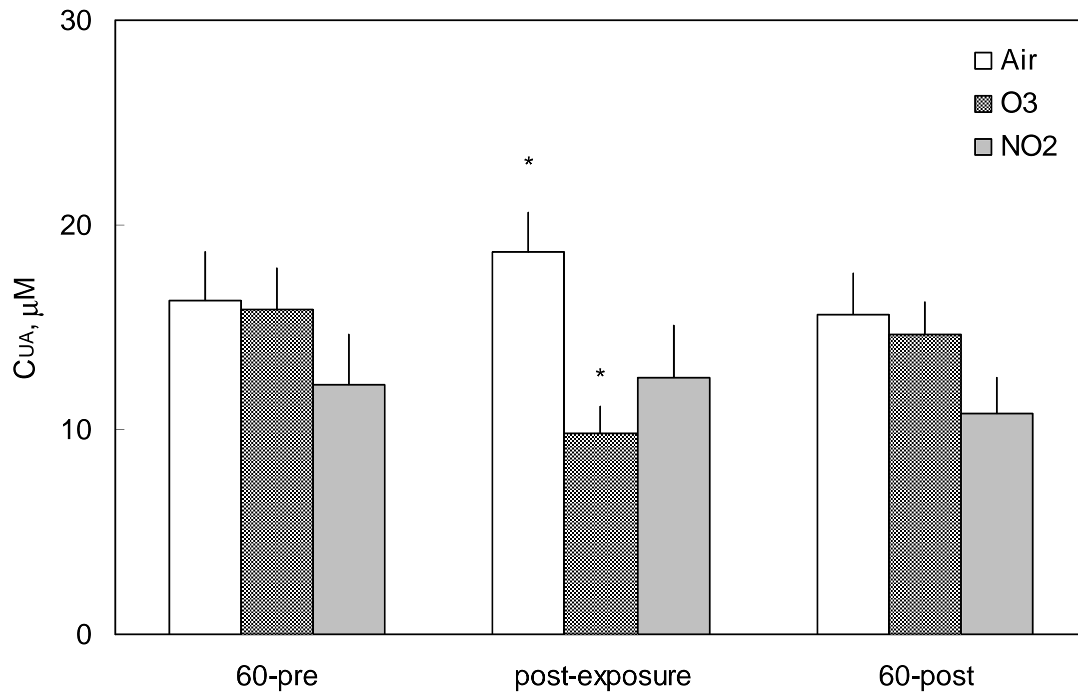


Figure 4.22. Mean±SE C_{UA} for air, O_3, and NO_2 exposure sessions for the twelve subjects that participated in protocol AF3. Asterisks indicate values are significantly different from 60-pre values.

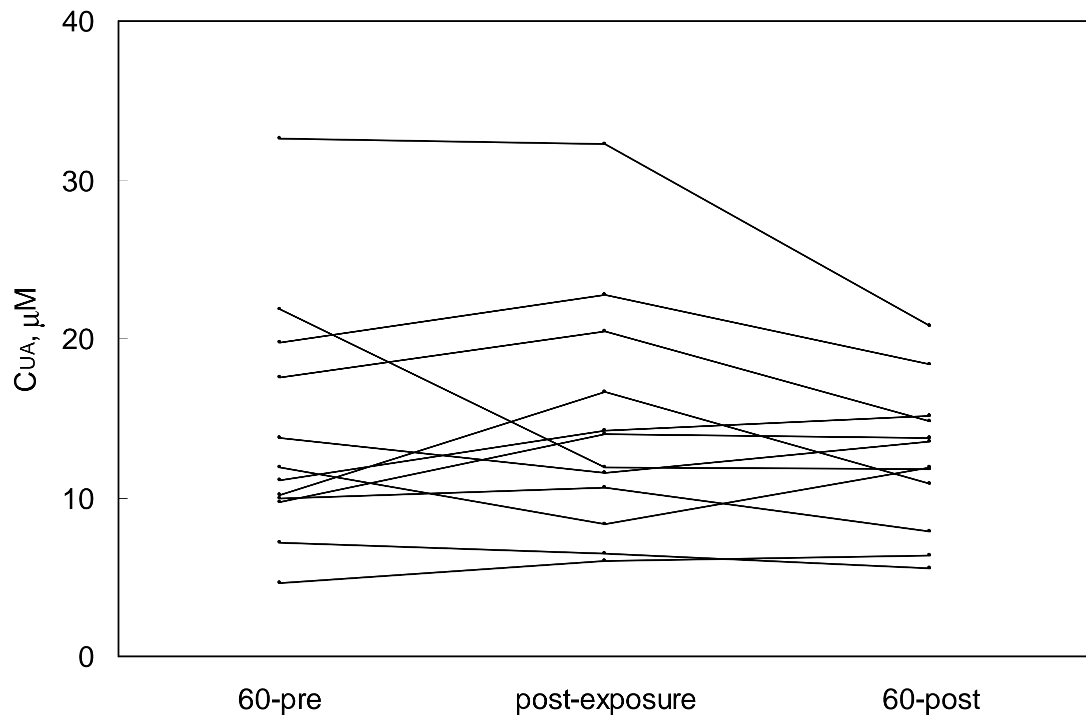


Figure 4.23. Trends in C_{UA} for each subject during NO_2 exposure sessions. Each line represents C_{UA} for one subject throughout the session.

4.3.3 Influence of Continuous NO_2 Exposure on Protein

Figure 4.24 shows mean±SE trends in protein levels for air, O_3, and NO_2, exposure sessions for ten subjects. One subject's protein data were not considered in the analysis because their protein levels were three standard deviations greater than the mean value for at least one data point. Another was excluded because protein levels were significantly higher than the bulk of this data throughout the session. Paired t-tests did not indicate any significant differences in protein levels between any of the sampling points ($p>0.4$). Individual trends in protein levels are shown below in figure 4.25.

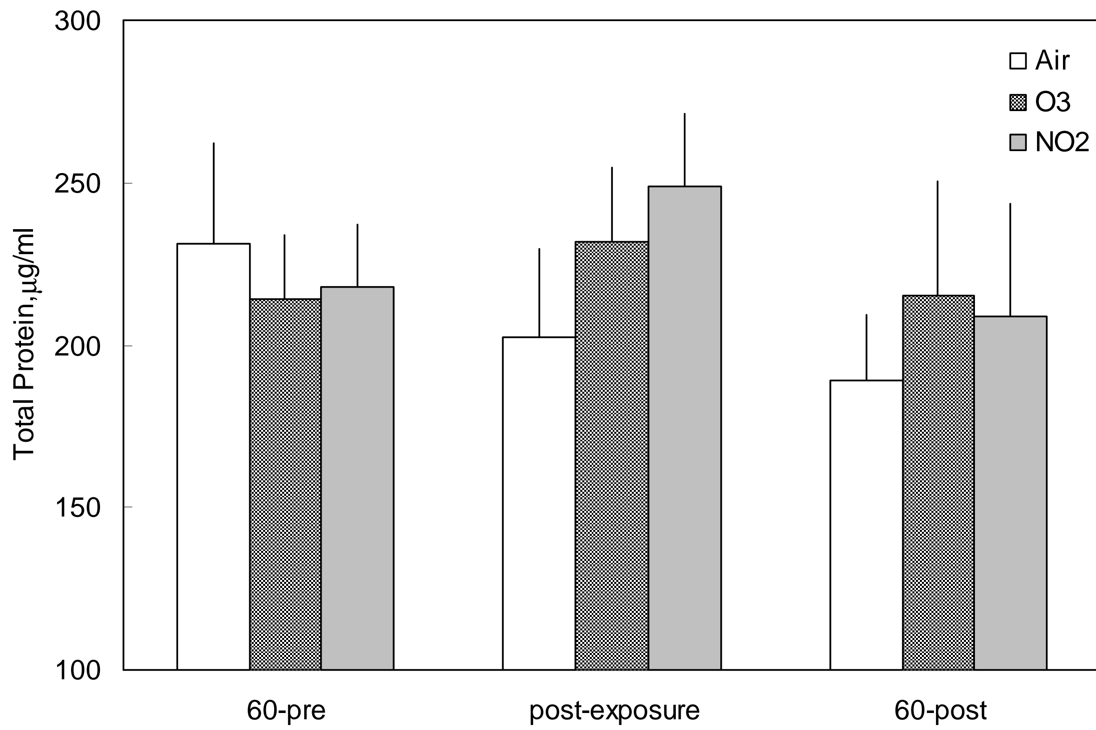


Figure 4.24. Mean±SE Total Protein for air, O_3, and NO_2 sessions for the subjects that participated in AF3.

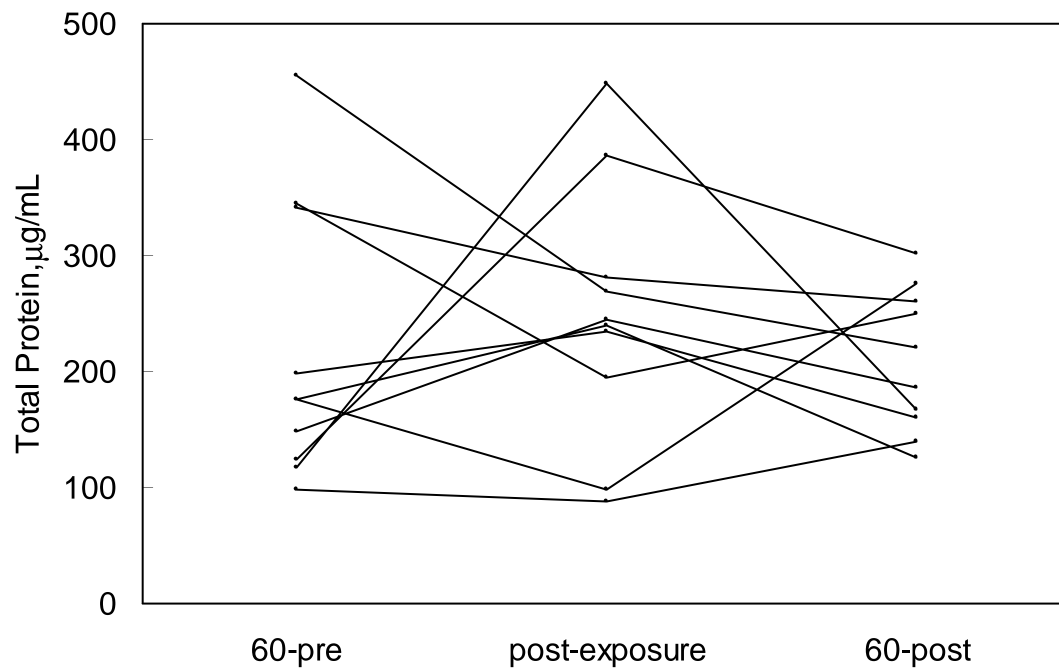


Figure 4.25. Trends in total protein for each subject in NO_2 exposure sessions. Each line traces total protein levels for one subject throughout the session.

4.3.4 Diffusion Reaction Modeling

Correlations resulting from variations in Λ and C_{UA} resulting from NO_2 exposure

Figure 4.26 relates Λ and C_{UA} for each subject before and after nasal NO_2 exposure. Each line segment connects two data points for Λ and C_{UA} acquired for each subject sixty minutes pre-exposure and immediately post-exposure to NO_2.

Conversion of data using eq 3.13 and plotting $-\ln(1-\Lambda)$ against $C_{UA}^{1/2}$ yields figure 4.27. ANCOVA performed on this data shows that there is no significant correlation ($p=0.103$).

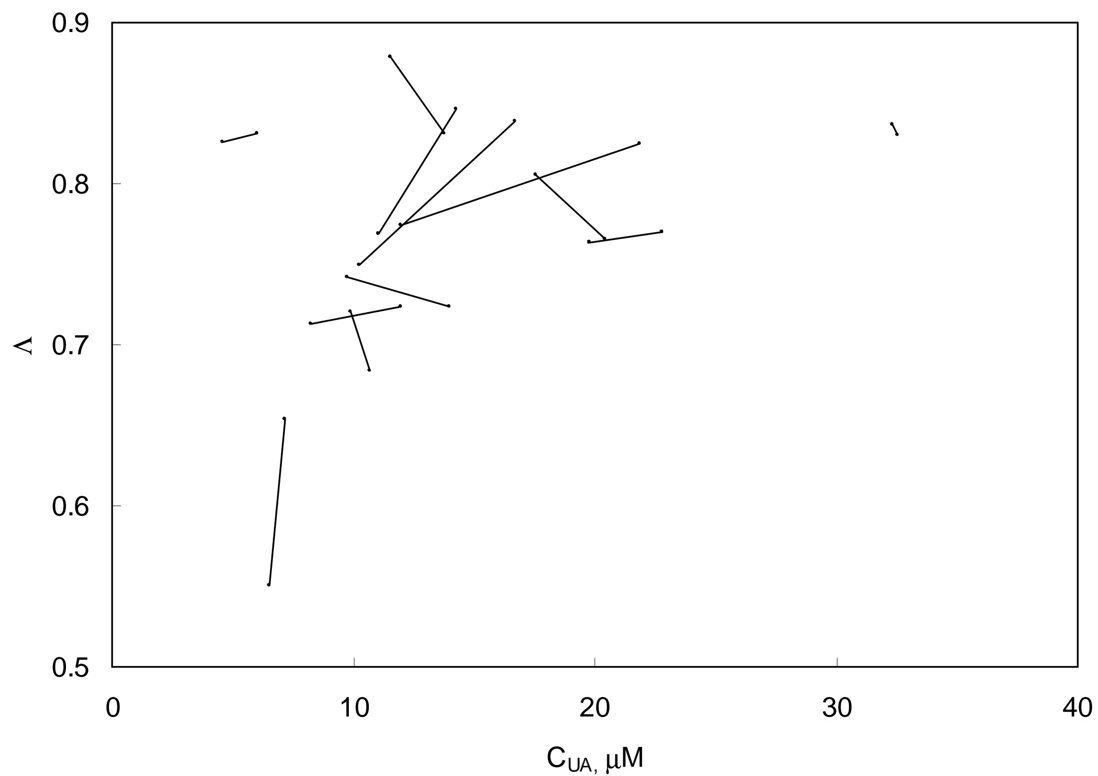


Figure 4.26 Relationship between Λ and C_{UA} measured at baseline and immediately post-exposure for each subject for NO_2 exposure sessions. Each line connects two data points obtained for one subject at the 60-pre, and post-exposure sampling points.

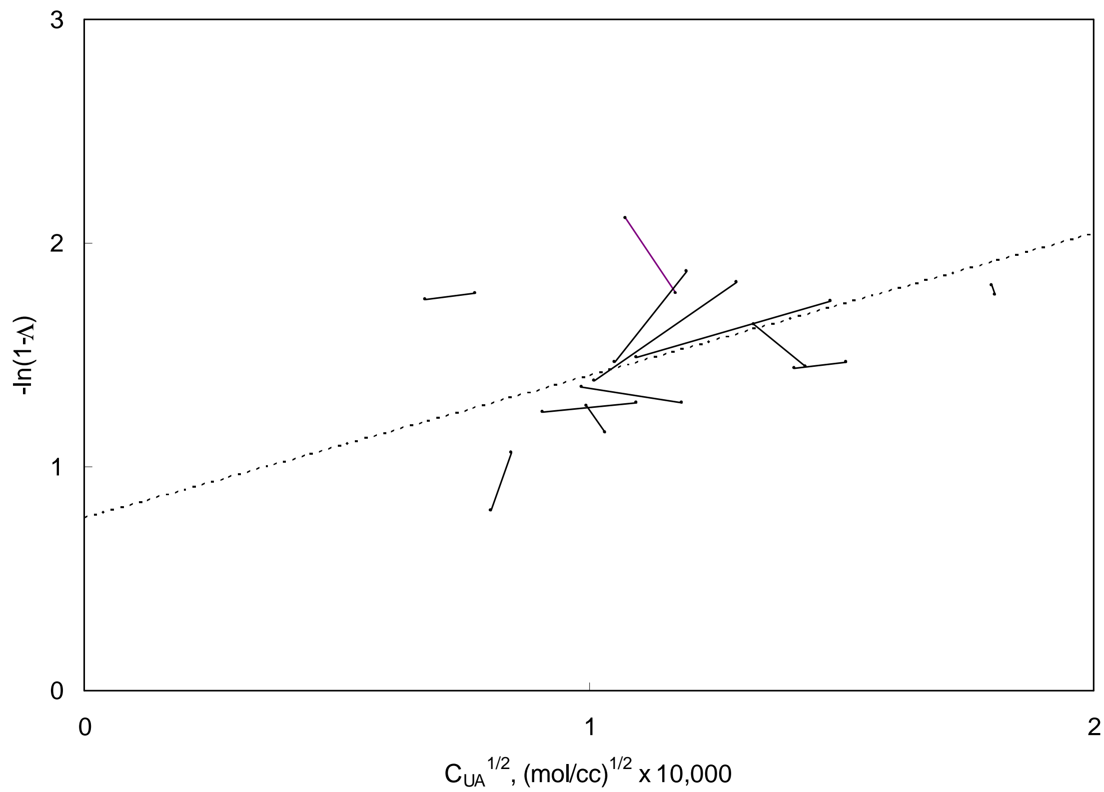


Figure 4.27 Relationship between $-\ln(1-\Lambda)$ and C_{UA} $^{1/2}$ for each subject before and after NO_2 exposure. Each line connects data obtained for one subject at the 60-pre and post-exposure measurements. The dashed line represents the regression for this data obtained from ANCOVA. The slope of this regression was insignificant (p=0.105).

4.3.5 TBARS response to O_3 exposure

Figure 4.28 shows mean±SE TBARS levels, in MDA equivalents, for the subjects enrolled in this study during air and O_3 exposure sessions.

Results of paired t-tests indicated that mean TBARS were not significantly changed throughout air exposure sessions ($p>0.3$). Initial comparison yielded insignificant responses in TBARS during O_3 exposures. However, removal of two data series that strongly conflicted with the trend resulted in the observation of a significant elevation in mean TBARS at each successive nasal lavage sampling point with significance levels at $p=0.011$ and $p=0.052$ at post exposure and 60-post exposure, respectively.

Individual subject trends in TBARS for the two sessions are provided in figure 4.29. It is seen that there were no consistent trends among air exposure sessions. In O_3 exposure sessions, eight of twelve subjects exhibited increased TBARS immediately post exposure, while nine of twelve subjects exhibited this trend 60 minutes post exposure.

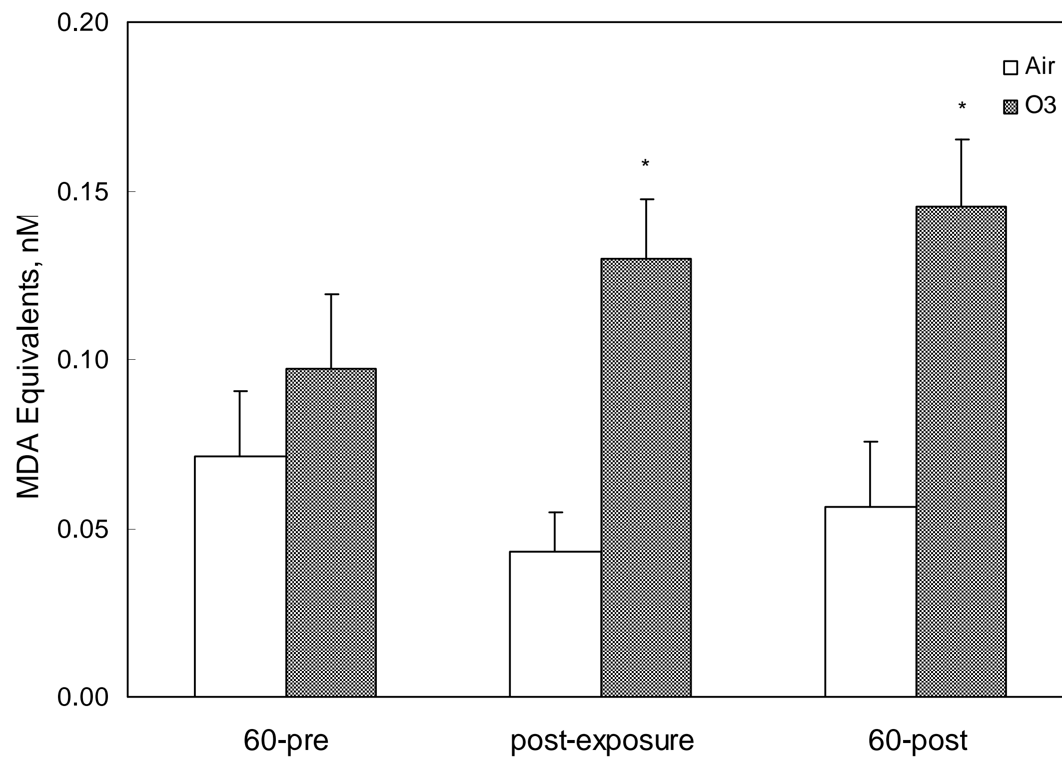


Figure 4.28 Mean TBARS±SE for air and O_3 exposure sessions. Asterisks indicate values are significantly different from pre-exposure.

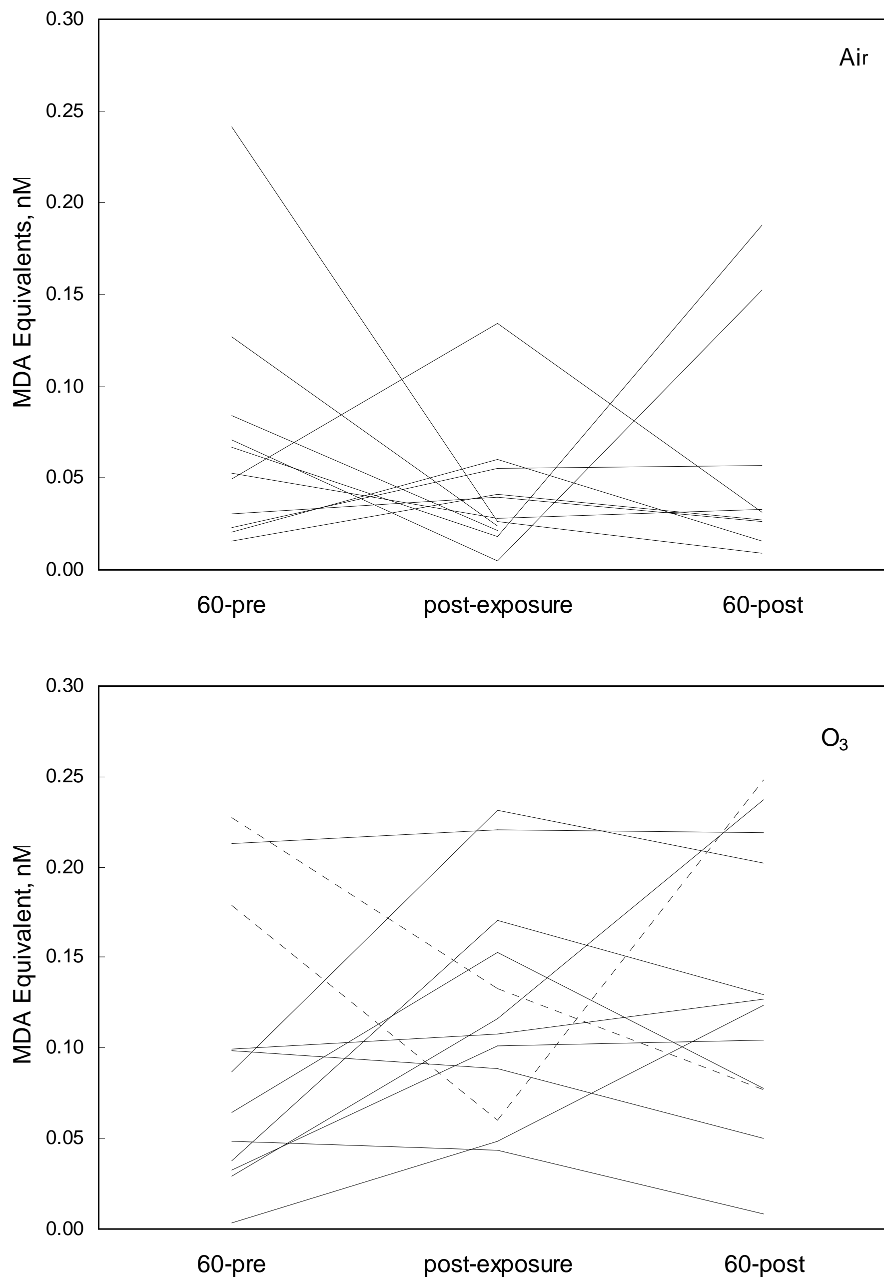


Figure 4.29 Trends in TBARS for each subject during air (top) and O_3 (bottom) exposure sessions. Each line represents TBARS measured as MDA equivalents for one subject throughout the indicated session. Data for two subjects that were removed from the analysis are indicated as dashed lines.

Chapter 5: Discussion

The goal of this work was to better understand the mechanism of ozone uptake in the respiratory system, using the nasal cavity as a model. Experiments were designed to determine the role of NLF uric acid levels in modulating fractional uptake of ozone in the nose. The apparent reactivity between O_3 and UA was estimated by incorporating experimental data with a reaction-diffusion model. In addition, we monitored the production of toxic ozonation products and the gaseous inflammation marker, nitric oxide, following ozone exposure.

One specific aim of this study was to determine the relationship between Λ and C_{UA}. We hypothesized that uric acid is the main contributor to O_3 uptake in the nose. To test this, chemical and physical perturbations were imposed on NLF UA levels of healthy human subjects such that several measurements of Λ and C_{UA} could be made for each subject within one day at varying levels of C_{UA}. The rationale for applying intra-day perturbations to UA levels, rather than studying its relationship with Λ on a day-to-day basis, was that there are significant day-to-day variations in the composition of NLF that may mask the observance of a correlation between Λ and C_{UA} (Santiago et al., 2001). Application of the data to a reaction-diffusion model allowed us to relate Λ

and C_{UA} to system parameters, and estimate the apparent reactivity between O_3 and UA. Once reactivity was known, we used the reaction-diffusion model to estimate the penetration depth of O_3 in the NLF.

Another aim of this work was to identify a toxicologically relevant marker of the nasal response to short term O_3 exposure. Several endpoints were evaluated before and after O_3 exposure. Gaseous NO was monitored in nasal air to indicate O_3 induced inflammation. TBARS levels in nasal lavage samples were measured to assess production of lipid peroxides and aldehydes, which are believed to be the primary carriers of O_3 induced toxicity to epithelial cells. Protein levels in NLF samples were monitored to track changes in epithelial permeability associated with inflammation. Nasal volume was measured to ensure that observed changes in Λ and C_{NO} did not result from O_3-induced geometric variation of the nasal airway caused by nasal congestion.

Studies involving human subjects were completed without any adverse effects. Lung function was not altered as a result of any research protocol. This was expected, considering exposures were administered to the nose during oral breathing, leaving little unreacted O_3 in the air stream capable of reaching lower airways. Unquestionably, the largest dose of exposure gas was delivered to the nose. However, subjects reported only trivial levels of nasal symptoms at the end of the research sessions.

5.1. Determination of the effect of continuous nasal O_3 exposure on subsequent O_3 uptake and NO production

The purpose of this protocol was to investigate the effect of continuous ozone exposure on subsequent ozone uptake. To restate, the uptake process occurs by a reactive diffusive mechanism whose rate is governed by antioxidant levels in NLF. The hypothesis of this study was that extended exposure of the NLF to O_3 depletes resident antioxidant levels, thereby lowering O_3 uptake. We also hypothesized that there would be an increased production of NO in the nasal tissues resulting from O_3 induced inflammation.

To test these hypotheses, protocol AF1 (Fig. 3.7) was applied to fifteen subjects. Ozone uptake was monitored once before, and several times following air or O_3 exposure during two separate sessions. Ozone exposure resulted in a significant reduction in Λ of 13.7% immediately following exposure ($p<0.001$), and 2.3% 15-minutes following exposure ($p=0.049$), whereas air exposure did not induce any changes (Fig. 4.1). The observation that uptake levels were reduced following O_3 exposure, but not air exposure, indicates that O_3 exposure induced depletion of NLF antioxidant levels. In other words, O_3 exposure created a situation where antioxidant depletion occurred at a faster rate than antioxidant regeneration. The finding that continuous exposure to O_3 reduces its subsequent fractional uptake has been observed in the human lung by Asplund et al. (1996). In their study, it was shown that fractional

O_3 uptake in the lung, measured by studying O_3 bolus penetration, was significantly reduced by about 5% following 2 h exposure to 0.36 ppm O_3 under resting conditions.

In our study, uptake began returning to baseline shortly after exposure was terminated (Fig. 4.1). Fifteen minutes following exposure, Λ was significantly higher than post-exposure values, but still lower than baseline values. There was no significant difference between Λ measured at baseline and 30-minutes post-exposure (p=0.228), or 60-minutes post-exposure (p=0.89), suggesting that Λ returned to baseline levels within the 30 minutes following termination of exposure. Similarly, Rigas et al. (1997) reported that in the 60 minutes following 2 hour exposure to 0.36 ppm O_3 in the human lung, O_3 uptake levels not only returned to baseline levels, but exceeded them.

As expected, C_{NO} did not exhibit a change in air exposure sessions. However, there was a small delayed increase in C_{NO} following O_3 exposure (Fig 4.3). Nitric oxide output was significantly increased by 7.3% 30-minutes post-exposure (p=0.022) and 5.8% 60-minutes (p=0.032) post-exposure, indicating an inflammatory presence. The delayed response of increased NO production was due to the slow nature of the inflammatory response, which peaks 4-6 hours following a disturbance (Schelegle et al., 1991). Because of this delay, other studies have not aimed to investigate NO responses within one hour following O_3 exposure. Production of increased NO over basal levels requires transcription of iNOS mRNA by inflammatory signaling molecules, followed by translation of the mRNA to the iNOS enzyme, which can take

several hours to occur (Asano et al., 1994). Therefore, we can conclude that the elevated NO concentrations observed in our study represented an early inflammatory response to O_3.

Our result offers the first evidence for increased gaseous NO generation in the nasal cavities following controlled, short-term O_3 exposure. Olin et al. (1999) reported that pulp mill workers who were occupationally exposed to O_3 had higher NO levels in exhaled air that an unexposed control group. However, in their study it was impossible to estimate the exposure level and to isolate effects of O_3 from those of other harmful compounds that may have been in the air. In a subsequent controlled study, Olin et al. (2001) reported that exposure to 0.2 ppm O_3 for 2 hours did not illicit an increase in NO levels in exhaled and nasal air. However, in their study, nasal air was sampled by sweeping air through the nasal cavity at a flow rate of 60 ml/min for 20 seconds. In their measurement, the total volume of gas displaced from the nasal cavity was less than a typical nasal volume of about 30 ml. Therefore, the flow rate of 60 ml/min may have been too low to adequately sample air from the entire nasal cavity. Additionally, because of the complex geometry of the nasal cavity, this low flow rate likely induces mixing effects resulting in a misrepresented measurement of NO concentration.

A unique property of NO is that it is very reactive with O_3 and may act as a gas phase antioxidant. However, ANCOVA failed to significantly predict uptake ($p=0.093$) using subject as a random factor and C_{NO} as covariate. Similarly, Santiago et al (2001)

applied a well mixed model to determine the gas phase contribution of NO to O_3 uptake, and concluded that there is a minimal effect.

Because the nasal cavity is highly vascularized, irritation of its epithelium may induce increased blood flow and capillary swelling, resulting in decreased nasal airway volume and altered surface area. Changes in these factors may influence uptake and NO levels. Therefore, nasal volume was measured to ensure that changes in these parameters occurred independently of changes in nasal geometry.

Nasal volume was significantly depressed immediately following air ($p=0.018$) but not O_3 ($p=0.066$) exposure (Fig 4.6). However, this significance was highly dependent on the data for only a few subjects. Upon examination of individual subject trends, there was no consistent difference in NV between air and O_3 exposure sessions. Most importantly, Λ and C_{NO} did not correlate with NV when comparing baseline measurements observed on the two experimental days. Prediction of uptake using an ANCOVA with subject as random factor and NV as covariate (Appendix E.5.1) yielded an insignificant correlation ($p=0.396$). A similar analysis predicting C_{NO} using an ANCOVA, with subject as random factor and NV as covariate (Appendix E.5.2), also yielded an insignificant correlation ($p=0.638$). Therefore, we can conclude that changes in Λ and C_{NO} did not result from changes in geometric parameters related to nasal volume.

The lack of response in NV to O_3 exposure may indicate that the system did not have time to illicit a physiological response. However, this is a convenient result, because changes in Λ and C_{NO} can be attributed to chemical and biological effects, respectively, rather than geometric effects.

5.2 Interpretation of Protocol AF2 - Determination of the influence of continuous O_3 exposure on C_{UA}, and the contribution of UA to Λ

The pilot study (AF1) indicated a pronounced lowering of ozone uptake following exposure to O_3. Because we hypothesize that UA is the main antioxidant contributor to O_3 uptake in the nose, the results of protocol AF1 would lead us to expect reduced uric acid levels following O_3 exposure. To test this, we monitored C_{UA} before and after O_3 exposure. We also hypothesize that Λ and C_{UA} are related according to Eq. 3.13.

Ozone exposure has been shown to enhance epithelial tissue permeability, measured by increased protein levels in lavage samples (Ishii et al., 2000). Increased permeability may enhance the flux of other compounds across the epithelium, such as systemic antioxidants including ascorbic acid. Therefore, NLF protein levels were measured to determine changes in tissue permeability that may influence O_3 uptake.

Another aim in this study was to determine if the exposure level was potent enough to illicit lipid peroxidation. We hypothesized that depletion of UA defenses by prolonged O_3 exposure would increase the lifetime of O_3 in the lining layer, and allow a greater

degree of interaction between O_3 and unsaturated fatty acids, yielding lipid peroxidation products.

It must be noted that experimental protocol AF2 was similar to that of AF1, except that a 60-minute pre-exposure measurement point was added to the beginning of the sessions. The rationale for adding one hour between the time of baseline measurements and exposure was to allow sufficient time for reconstitution of NLF constituents that were washed away in the initial nasal lavage; Santiago showed that Λ and C_{UA} levels return to baseline within an hour following nasal lavage.

Trends in Λ were similar to those in the pilot study. Air exposure did not induce a change in Λ immediately post-exposure compared to 60-pre- and immediately-pre-exposure values. However, there was a small yet significant ($p=0.034$) depression in Λ of 2.4% 15-minutes post air exposure compared to immediately pre-exposure. This was likely a result of lavage performed 15 minutes prior to the uptake measurement, which temporarily removed resident reactive compounds from the NLF. The fact that uptake was not significantly different from post-exposure values at 30-minutes or 60-minutes following air-exposure falls within Santiago's (2001) findings suggesting that uptake returns to pre-lavage levels 30 minutes following lavage.

A reduced uptake of 18.9% was observed immediately post O_3 exposure. The depression in Λ 15-minutes post-exposure of 3.4% was likely due to the combined

effects of O_3 exposure and subsequent nasal lavage. Nonetheless, uptake returned to baseline levels 30-minutes following O_3 exposure, indicating that the NLF regenerates baseline levels of antioxidants following the combination of exposure and lavage challenges.

Three lavage samples were taken during each session at 1) 60 minutes prior to exposure, 2) immediately post exposure, and 3) 60 minutes post exposure. Interestingly, C_{UA} in the samples were significantly ($p=0.028$) elevated by 4.8% immediately following air exposure. This may have been part of a physiological feedback response triggered by nasal lavage, which disrupted NLF oxidant-antioxidant balance by removing resident antioxidants. Increased C_{UA} following air exposure may also have occurred if the zero-air source used as the exposure gas during the air sessions contained less trace oxidative compounds than room air. If this was the case, C_{UA} levels should increase to a new level following 30-minutes of air exposure. This theory is supported by the finding that C_{UA} was elevated following 30-minutes of air exposure, but returned to baseline after breathing room air during the hour following air exposure.

Uric acid concentration was significantly ($p<0.001$) decreased by 30.2% following O_3 exposure, indicating that O_3 does in fact undergo measurable reaction with uric acid in vivo. C_{UA} returned to baseline 60 minutes following termination of exposure, proving that uric acid levels were fully restored following consecutive O_3 exposure and nasal

lavage challenges. This result is consistent with work done by Mudway et al (1999), which showed that uric acid levels in nasal lavage were similar 60 minutes following a 2 hour exposure to either 0.2 ppm O_3 or pure air. Their work also suggested that UA released into the NLF originated in the mucosal glands for the first hour of exposure because plasma UA levels were unchanged in that time period. Conversely, in their second hour of exposure, significantly higher UA levels were observed in the plasma, suggesting the possibility of a systemic response responsible for repletion of UA stored in nasal mucosal glands. Because the exposure conditions used in our study delivered less O_3 than Mudway's study did in their first hour of exposure, we conclude that UA levels observed in our experiments were primarily sourced in local glandular stores.

Protein levels were not significantly changed from baseline immediately following air or O_3 exposure. There was a barely significant reduction of 10.3 % in protein levels one hour following exposure to ozone. Upon inspection, this tend was observed in about half the subjects for both air and O_3 sessions and was most likely due to washout of protein from nasal lavage sampling. It is expected that reconstitution of NLF protein levels takes longer than that of antioxidant compounds, such as UA, because of their larger size and non-localized origin. Indeed, Santiago (2001) showed that during normal quiet breathing, protein levels were slightly (but insignificantly) reduced one hour following nasal lavage. Although we had initially expected protein levels to increase in response to inflammation (Schelegle et al., 1991; Ishii et al., 2000), the time scale of our experiments may not have been long enough to observe this effect. In any

case, the overall result is that protein levels were not changed, suggesting that there was no change in permeability because of O_3-induced tissue inflammation.

In this study, O_3 uptake was attributed to reaction with uric acid only. To test the contribution of protein levels to Λ, ANCOVA was used to predict uptake with subject as a random factor and protein as a covariate for data taken at baseline and immediately post-exposure (Appendix E.5.4). This produced an insignificant correlation ($p=0.239$) indicating that protein levels are not a predictor for ozone uptake. This suggests that, although proteins are susceptible to oxidation, they do not significantly influence ozone uptake. This result is in agreement with Mudway and Kelly's (1998) finding that continuous O_3 exposure did not influence protein concentration in bronchoalveolar lavage.

To evaluate the contribution of uric acid to uptake, baseline data from the two days was converted according to eq. 3.13 to yield figure 4.15. ANCOVA performed on these values indicated no significant correlation when attempting to predict uptake using subject as a random factor and C_{UA} as a covariate ($p=0.185$). This lack of correlation is consistent with findings by Santiago which investigated the relationship between uptake and C_{UA} on a day-to-day basis. In their study a components of variance analysis showed that there was significant day-to-day variability with the two factors, subject and day, each accounting for about half of the total variability in Λ. These day-to-day

variations may result from subtle concentration differences in compounds other than UA, and may mask the observation of a relationship between Λ and C_{UA}.

In this protocol, day-to-day variability was eliminated by observing simultaneous changes in Λ and C_{UA} on a same-day basis. This was done by employing continuous O_3 exposure to alter NLF UA composition. Comparison of data taken 60 minutes before and immediately after O_3 exposure was applied to Eq. 3.13, and produced a highly significant ($p<0.001$) correlation (Fig 4.19). The slope of this regression was 13776±3181 $M^{-1/2}$ and was used to calculate (Eq. 3.14) the apparent second order reaction rate constant between O_3 and C_{UA}, k_2, which was $1.56 \times 10^9 \pm 0.74 \times 10^9\ M^{-1}s^{-1}$. A discussion of the observed value for k_2 is presented later in this chapter (Section 5.5). An important finding was that the intercept of regression was not significantly different from zero, indicating that mass transfer cannot occur across the phases in the absence of uric acid. This is in agreement with our assumption that uric acid is the main contributor to uptake in the nasal cavity.

5.3 Interpretation of Protocol AF3 - Effect of NO_2 exposure on Λ and C_{UA}, and determination of TBARS formation following O_3 exposure

One purpose of this protocol was to determine the role of C_{UA} in modulating Λ by using nitrogen dioxide (NO_2) exposure to illicit a perturbation in uric acid levels. Because NO_2 is an oxidative gas, it is logical to expect that prolonged exposure would deplete NLF UA stores and reduce successive uptake values. However, Rigas et al. (1997)

demonstrated that exposure to 0.36 ppm NO_2 in the lung results in an increased level of uptake, suggesting that NO_2 may trigger the release of antioxidants.

We used 1.0 ppm NO_2 as the exposure gas. This concentration was chosen based on previous studies that administered safe levels of NO_2 and induced a noticeable response in subjects (Hackney et al, 1978). It must be noted that NO_2 levels used in this study are a small fraction of limits set by ACGIH, whereas the O_3 levels used are three times greater. However, few studies have previously exceeded 1 ppm NO_2 exposure, and we adopted that level for safety concerns.

We found that exposure to NO_2 induced a small, yet significant ($p=0.049$) reduction in uptake of 3.2% following NO_2 exposure. This change was significant only when comparing immediately pre-exposure to post-exposure values of Λ, and not when comparing baseline (60-pre) to immediately post-exposure. This result supports our hypothesis that NO_2 exposure would illicit decreased O_3 uptake, suggesting that NO_2 has a net oxidative effect in the nose. Our result is contrary to findings by Rigas et al (1997) which observed increased O_3 uptake in the lung following low level exposure to NO_2. However, in the same study, this effect was not observed at exposure to higher NO_2 levels. Therefore, the increased uptake following NO_2 exposure observed by Rigas et al may have been due to the same clean air exposure effect observed in our study's air exposures, which resulted in increased UA (Fig. 4.10).

Changes in C_{UA} did not accompany the slightly decreased uptake, implying that although NO_2 is an oxidative compound, there is a minimal rate of reaction between NO_2 and C_{UA} in the nose. This raises the question: Does NO_2 undergo appreciable reactivity with UA in vitro? Relatively little data has been reported on NO_2 reactivity with antioxidants. Therefore, to answer this question, we investigated NO_2-antioxidant interactions in an in vitro liquid film system (Kermani at al. 2006). In this experiment, liquid films of solutions containing known concentrations of about 200 μM of the antioxidants UA, AA, and GSH were exposed to 1.0 ppm O_3 or 1.0 ppm NO_2 for 10 minutes. The depleted amount of each antioxidant was determined by HPLC to compare reactivity of each compound with O_3 and NO_2. The results are shown in figure 5.1.

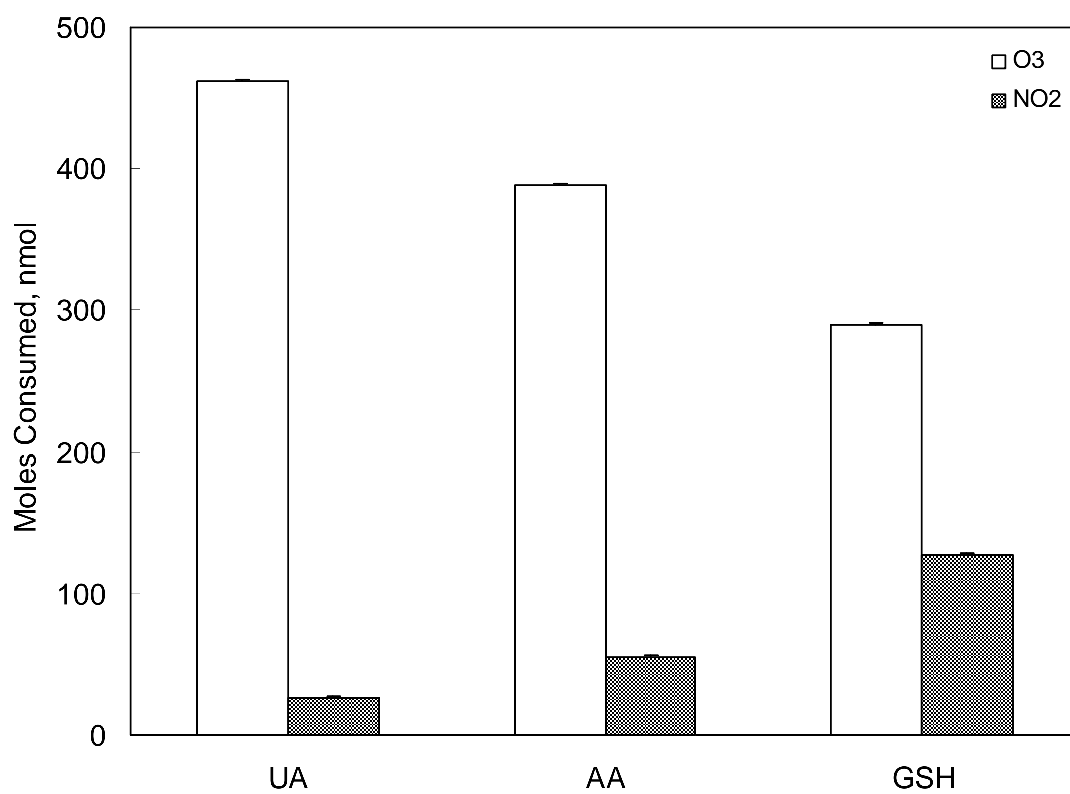


Figure 5.1. Antioxidant consumption in 200 μM liquid film solutions exposed to 1.0 ppm O_3 (clear bars) or 1.0 ppm NO_2 (filled bars) for 10 minutes.

Between UA, AA, and GSH, it was found that UA was the least preferred oxidation target of NO_2. Indeed, O_3 was much more reactive than NO_2 with all antioxidants studied, especially UA. It must be noted that in human studies experiments, we used 0.36 ppm O_3, not 1.0 ppm as in the in vitro study. Therefore, the data obtained from the in vitro study does not exactly represent antioxidant depletion at exposure conditions in the nose. Nonetheless, there was an 18-fold greater depletion of UA resulting from 1.0 ppm O_3 exposure than from 1.0 ppm NO_2 exposure in the model solutions.

Although NO_2 has the limited ability to oxidize NLF compounds, it apparently did not appreciably oxidize any NLF substrates that modulate ozone uptake (4.22). The facts that 1) NO_2 did not appreciably react with UA in vitro, and not at all in vivo, and 2) that there was only a minimal reduction in uptake, are in line with our hypothesis that UA is the major contributor to O_3 uptake in the nose. The considerable oxidation of GSH by NO_2 in model solutions but lack of appreciable change in ozone uptake following NO_2 exposure is in agreement with previous literature suggesting that GSH plays a minimal role in governing ozone uptake in the nose (Housley at al, 1995)

Protein levels were not significantly changed in this protocol, suggesting that exposure may have been too mild, or that antioxidants remained at levels sufficient to inhibit protein-NO_2 reactions from diminishing protein levels. The fact that protein levels did not change throughout the session also suggests that there were no increases in epithelial permeability that would have increased protein levels.

The second purpose of this protocol was to investigate the occurrence of O_3 induced lipid peroxidation. Under normal conditions antioxidants provide protection against toxic effects of O_3. However, in situations where gaseous O_3 concentration is high, or when antioxidant levels have undergone significant depletion, O_3 may penetrate deeper into tissue and have an extended lifetime before reacting with substrates. In this situation, non-antioxidant compounds, such as unsaturated fatty acids, may be oxidized at appreciable rates, leading to the formation of toxic compounds such as lipid peroxides and aldehydes.

The formation of these compounds was collectively assessed by monitoring levels of thiobarbituric acid reactive substances (TBARS). Removal of three visual outliers yielded the observance of significantly elevated TBARS levels ($p=0.01$) immediately following exposure to O_3 (Fig. 4.29), suggesting significant formation of lipid oxidation products. TBARS levels were subsequently further increased 60 minutes following termination of exposure, indicating prolonged free radical activity. Intuitively, it is expected that lipid peroxidation should cease following the lavage conducted immediately following exposure. However, the aqueous saline solution may not have extracted all of the organic lipid peroxides, thus promoting further activity.

To evaluate the role of UA in inhibiting lipid peroxidation, we constructed a plot of TBARS levels in nasal lavage immediately following O_3 exposure against C_{UA} at the beginning of the session (Fig. 5.2). A regression performed on the data suggests that,

with the exception of a few points, there was a significant relationship ($p<0.001$) between TBARS levels following exposure and C_{UA} prior to exposure. In other words, subjects that had higher levels of C_{UA} at the beginning of the session produced less lipid peroxidation products during exposure to O_3. This result is remarkable, considering the possibility of daily variations in many variables, such as levels of NLF unsaturated lipid compounds.

Upon further inspection, we aimed to relate changes in TBARS levels to initial uric acid concentrations. Differences in TBARS levels from 60-pre to immediately post-exposure time points were plotted against baseline (60-pre) C_{UA} levels (Figure 5.3). The result obtained was less compelling than that in figure 5.2. Two subjects displayed reduction of TBARS levels following O_3 exposure, which may have been due to high baseline levels due to various reasons such as mild allergy or residual inflammation due to illness. Two subjects showed no change in TBARS levels, one of which had high C_{UA} levels that could have inhibited TBARS formation altogether. With the exception of two of the three visual outliers identified in figure 5.2, the rest of the data points follow the expected trend, which states that higher UA levels should inhibit TBARS formation. However, only seven of twelve subjects follow this rule. As a result, it appears that C_{UA} in the NLF modulates absolute TBARS levels following exposure (Fig. 5.2), rather than differences in them from pre- to post- exposure (Fig 5.3).

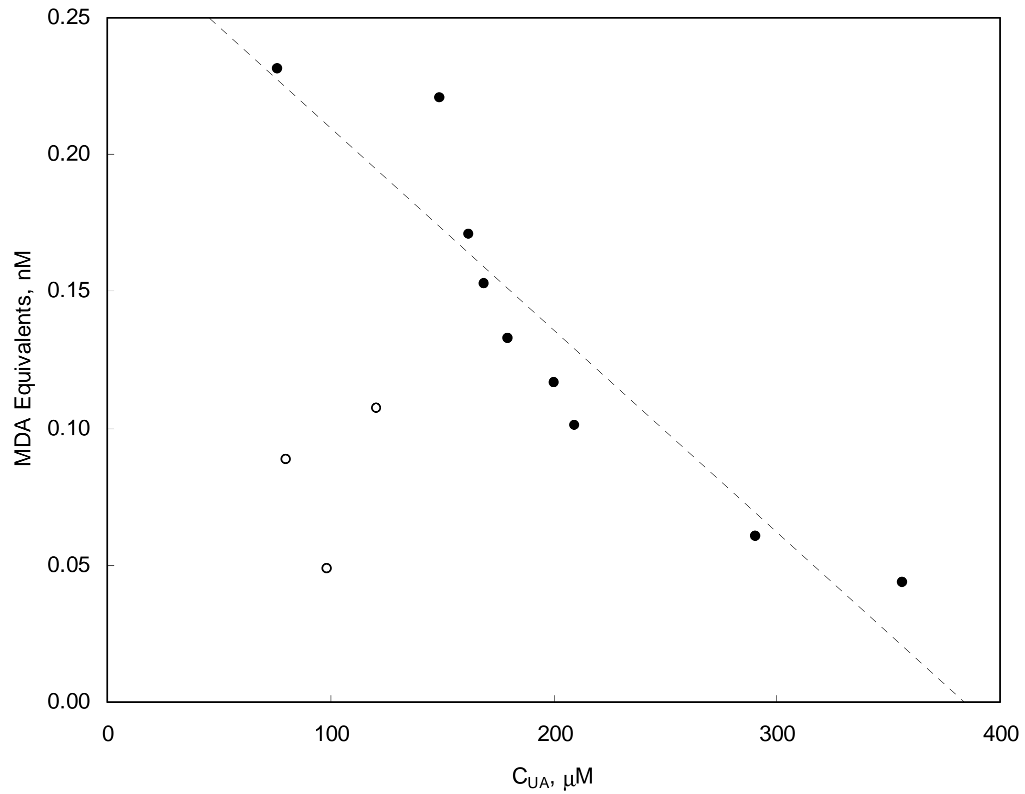


Figure 5.2 Uric acid inhibition of TBARS formation. Each point indicates TBARS levels following O_3 exposure and UA levels prior to O_3 exposure for one subject. Solid circles represent data used in the regression. The dashed line represents the regression line. Open circles indicate visual outliers.

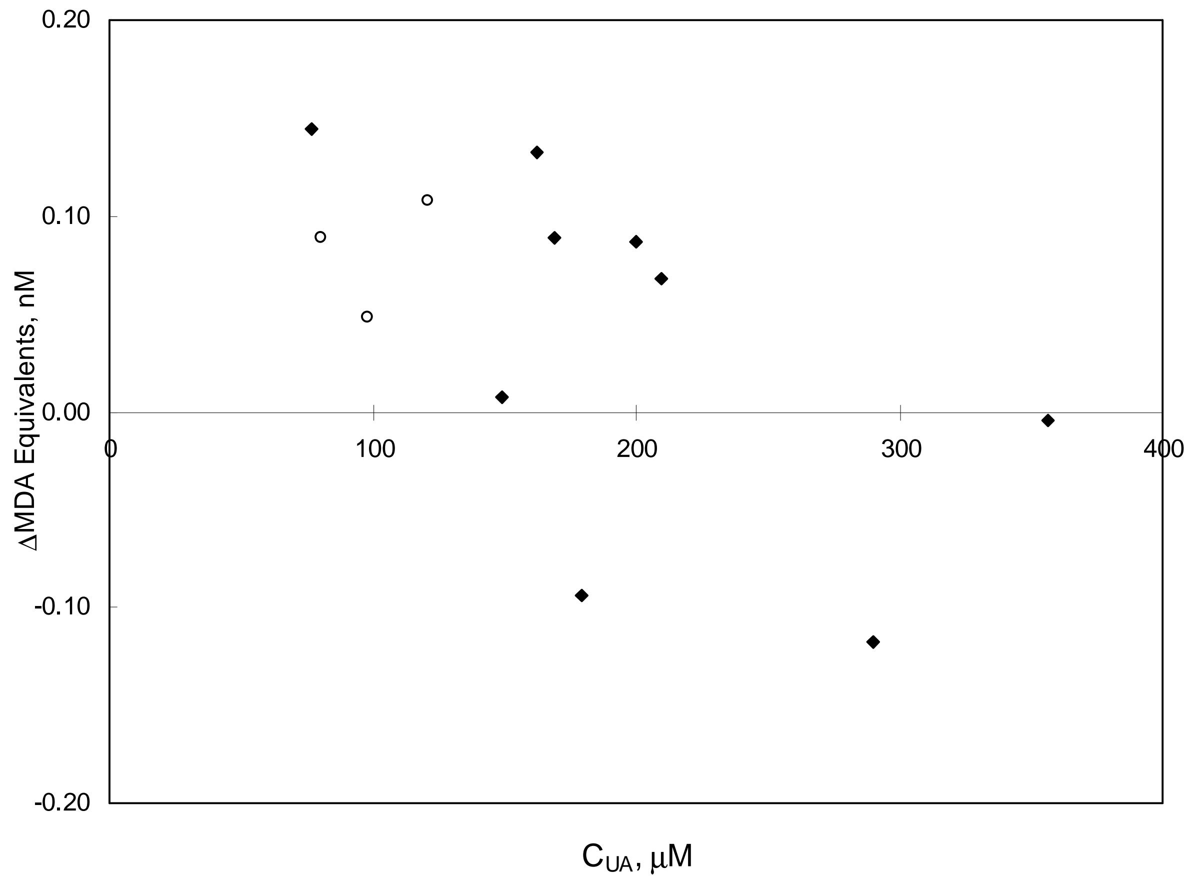


Figure 5.3 The relationship between changes in TBARS levels and C_{UA}. Each point relates the difference between TBARS from 60-pre to immediately post O_3 exposure to C_{UA} levels at 60-pre, for one subject. Open circles represent visual outliers identified in figure 5.2.

5.4 Interpretation of Protocol LS1 – Removal of Uric Acid by Subsequent Nasal Lavage

Previous work by Santiago (2001) provided data that is useful to this study because it can be applied to investigate the relationship between Λ and uric acid on a same day basis. In their study, the nasal cavities were consecutively washed out with saline once every minute for three minutes using the nasal lavage technique described in Chapter 3. It was observed that performing nasal lavage washes away resident antioxidants from the NLF, thus reducing uptake. The experimental protocol and raw data for their study is provided in Appendix D.

Nasal lavage induced a lowering of uptake at each subsequent sampling time. This lowering of uptake was statistically significant after the first lavage ($p<0.001$), but not the second ($p=0.139$). On the other hand, uric acid levels were significantly decreased following each washout ($p<0.002$). The data indicates that both uptake and uric acid levels are influenced by nasal lavage. These findings can be conveniently applied to investigate the relationship between uptake and uric acid on a same day basis.

Using Eq 3.13 we create a plot of $-\ln(1-\Lambda)$ against $C_{UA}^{1/2}$. An ANCOVA performed on these data, significantly ($p<0.001$) predicted $-\ln(1-\Lambda)$ using subject and $C_{UA}^{1/2}$ as random factors and covariates, respectively. The slope of the regression was $2978.6\pm422.5\ M^{-1/2}$. Using equation 3.14, we calculated a k_2 value of 6.04×10^8

$\pm 1.71 \times 10^8$ $M^{-1}s^{-1}$. An interesting finding resulting from this analysis is that the intercept of the regression is significantly nonzero ($p<0.001$), indicating that uptake does occur across the gas and NLF phases in the absence of UA.

Nasal lavage is a rapid method for removal of antioxidants from the NLF. Because the body is a highly adaptive system, it may possess a physiological process that quickly responds to this acute induction of oxidant stress by rapid local release of antioxidants to the NLF. The significant non-zero intercept of the consecutive lavage study may be explained by this phenomenon if a substantial amount of compounds other than UA were secreted into the NLF following lavage.

Another possible explanation for non-zero uptake in the absence of uric acid is that serial nasal lavage maneuvers may have removed the nasal lining layer altogether, leaving underlying cell membranes exposed to react with ozone. This theory is supported by data that shows that O_3 can react with cell membranes of red blood cells to liberate acetylcholinesterase (Ballinger et al, 2005). Constituents of cell membranes also include proteins, phospholipids, and cholesterol which may be susceptible to ozonation (Uppu et al., 1995).

Finally, each successive nasal lavage maneuver removes water soluble antioxidant compounds, but leaves lipid soluble compounds behind in the nose. Although the contribution to overall uptake of lipid compounds associated with NLF is small, the

relative effect of lipids accumulates with each successive nasal lavage, and is likely some component of the non-zero intercept observed here. In contrast, this effect was not observed in O_3 exposure perturbation experiments because ozone-reactive lipids were likely depleted via attack by ozone.

5.5 Comparison of Regression Parameters and k_2's Obtained from Protocols AF2 and LS1

In this research, we utilized data obtained from NLF perturbation studies, and determined the apparent second order reaction rate constant, k_2, between O_3 and UA, for each study. Several perturbations were carried out, but two methods yielded a significant alteration in C_{UA} and Λ that were related by Eq. 3.13: 1) continuous nasal O_3 exposure, and 2) consecutive nasal lavage sampling. Values of k_2 obtained from in vivo nasal experiments were in the range of 10^8-10^9 (Table 5.1).

Perturbation Method	Slope, $(cc/mol)^{-1/2}$	p-value$_{slope}$	k_2, $M^{-1}s^{-1}$
O_3 Exposure	13376±3181	<0.001	$1.56x10^9 \pm 0.74x10^9$
Consecutive Nasal Lavage	2978.6±422.5	<0.001	$6.04\ x10^8 \pm 1.71\ x10^8$

Table 5.1 Comparison of regression slopes (Eq. 3.14) and k_2 obtained from protocols AF2 and LS1. Errors indicated represent standard error.

Previously reported values of k_2 in the literature were determined by studying the reaction between O_3 and model uric acid solutions. Typical values for k_2 obtained in these studies range from 10^6-10^7 M^{-1} s^{-1} (Kermani et al., 2006). Indeed, the k_2 values

obtained in our study are significantly greater than previously reported values. However, the values obtained from our perturbation methods are in close agreement with each other as well as k_2 inferred from other in vivo experiments. Santiago et al. (2001) estimated a quasi first order reaction rate constant of $2.5x10^5$ s^{-1} for O_3 by studying ozone uptake at various flowrates in the human nose. Assuming an average C_{UA} in the nasal lining layer of 100 μM, we estimate a second order reaction rate constant between O_3 and UA of $2.5x10^9$ $M^{-1}s^{-1}$. This value is within the range of those obtained in our work. In a study involving O_3 bolus penetration in the lungs via nasal breathing, Bush et al. (2001) calculated a first order rate constant of $8x10^6$ s^{-1}, which corresponds to a second order rate constant of $8x10^{10}$ $M^{-1}s^{-1}$ between O_3 and UA, when C_{UA}=100 μM. This value is an order of magnitude higher than found in our study. This positive difference most likely arises because ozone reacts with antioxidants in the lower airways in addition to those in the nose during bolus measurements.

An important difference between regressions resulting from exposure and washout studies was they had different levels of significance for the intercept (Table 5.2). The correlation resulting from O_3 exposure indicated that the intercept was not significantly different from zero (Fig. 4.19), suggesting that mass transfer can not occur in the absence of UA. This result validates our assumption that UA is main contributor to ozone uptake in the nose.

Perturbation Method	Intercept	p-value	Uptake (C_{UA}=0)
O_3 Exposure	-0.350±0.390	<0.441	-
Saline Washout	0.276±0.039	<0.001	0.24±0.08

Table 5.2 Comparison of y-intercepts and corresponding Λ obtained from protocol AF2 and LS1.

However, the intercept arising from the regression obtained in the consecutive lavage study was significantly non-zero (Fig. D.2), indicating that O_3 uptake can occur in the absence of UA. The value for the intercept of this regression was 0.276±0.039. Consequently, this is the value for the left hand side of Eq. 3.13, -ln(1-Λ), when C_{UA}=0. A simple calculation reveals that this intercept corresponds to a Λ value which is 0.24±0.08. Comparison of this value to the mean pre-exposure Λ value of 0.462, we see that UA accounts for about half of the total uptake, following subsequent nasal lavage procedures. A discussion of possible reasons for a non-zero intercept can be found in section 5.4.

The difference in the y-intercepts of regressions presented in figures 4.19 and D.2 were likely due to the differences in perturbation method. On one hand, continuous ozone exposure selectively depleted uric acid levels while leaving the NLF physically in tact. On the contrary, subsequent nasal lavage removed NLF constituents, leaving cell membranes exposed.

As stated before, application of the reaction-diffusion model (section 3.4) to data obtained from our experiments only yielded a significant result for k_2 in continuous O_3 exposure and subsequent nasal lavage experiments. Application of Λ and UA resulting from day-to-day variations (fig. 4.15), and NO_2 exposure (fig. 4.29), did not yield significant a correlation between $-\ln(1-\Lambda)$ and $C_{UA}{}^{1/2}$. However, it is worth stating that the slopes of these statistically insignificant regressions were positive indicating that data follows the intuitive trend.

5.6 Evaluation of O_3 Penetration Depth in the NLF

To investigate the penetration distance of O_3 into the NLF, we generated a plot of the O_3 concentration profile in the NLF using Eq 3.5. The value of k_2 used to predict the penetration profile was 10^9 $M^{-1}s^{-1}$, which represents a rough average of values obtained in O_3 exposure and subsequent lavage perturbation studies. Uric acid concentration in the lining fluid, adjusted for dilution, was assumed to be 200 μM. This value was based on pre-exposure C_{UA} values for subjects participating in protocols AF2 and AF3. The NLF thickness, L, was conservatively chosen as 1 μm. The O_3 concentration profile in the NLF is displayed in figure 5.4.

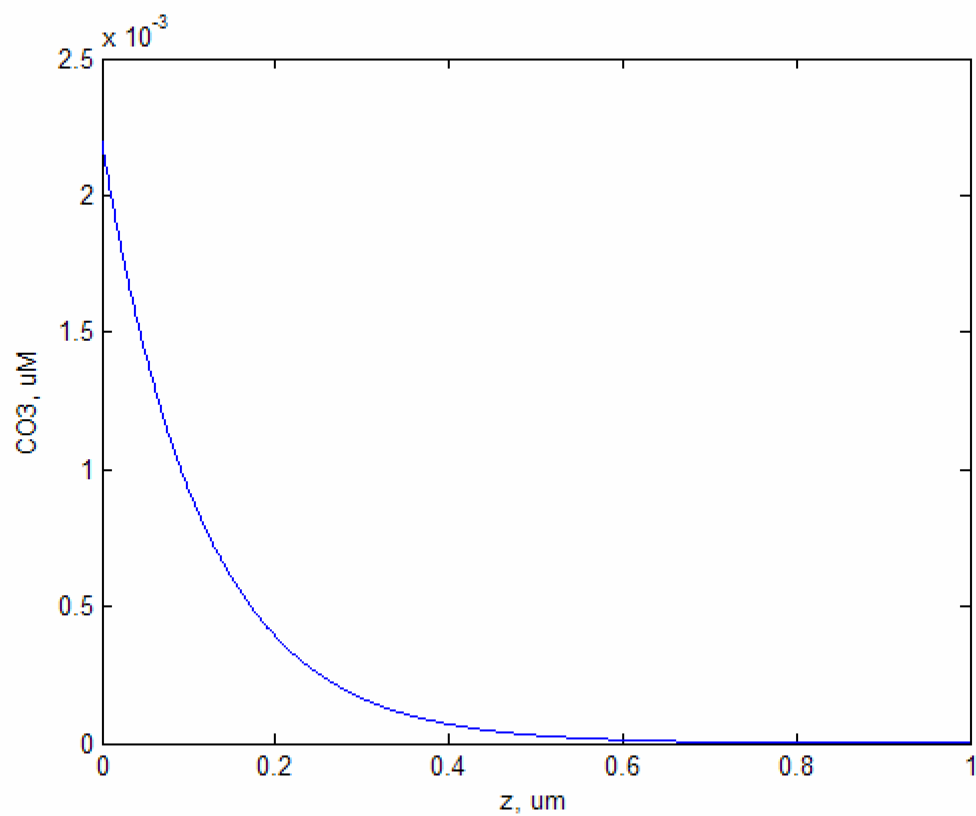


Figure 5.4. Predicted O_3 concentration profile in the NLF assuming $k_2=10^9$ $M^{-1}s^{-1}$, C_{UA} in the NLF=200 μM, and NLF thickness=1 μm.

Figure 5.4 indicates that O_3 penetrated to a depth of about 0.6 μm past the NLF surface, which was greater than the estimate of 0.1 μm reported by Pryor (1992). In his work, penetration depth was estimated in the lungs by comparing the diffusion time of O_3 across a 0.1 μm thick RTLF layer to its reaction half-life in the RTLF. To calculate the half life of O_3, Pryor assumed that it reacts solely with glutathione at an assumed concentration of 1 mM, and second order reactivity of 10^9 $M^{-1}s^{-1}$. However, the assumed values for both of these parameters were much higher than those determined experimentally in the literature. Hatch estimated glutathione concentration in the lung at 100 μM, which is one tenth the value assumed by Pryor. Kanofsky and Sima observed a second order reaction rate constant of 2.5 x 10^6 $M^{-1}s^{-1}$ between model GSH solutions and gaseous O_3. This value is three orders of magnitude less than that assumed by Pryor. As a result of overestimation of reaction parameters, Pryor underestimated the O_3 penetration distance into the NLF.

The pseudo first-order rate constant used in Pryor's analysis was $k_{2,g}C_{gluthathione}= 10^9 \text{ x } 10^{-3} = 10^6 \text{ s}^{-1}$, where $k_{2,g}$ represents the second order rate constant between O_3 and glutathione, and $C_{glutathione}$ represents the concentration of glutathione in the NLF. In contrast, the pseudo first order rate constant used in our work was $k_2C_{UA}=10^9 \text{ x } 200\text{x}10^{-6} = 2\text{x}10^5 \text{ s}^{-1}$. As a result, Pryor's estimate of the first order reaction rate constant was 5 times greater than ours, explaining why we observed a greater penetration depth of O_3.

Given that the nasal lining layer thickness is about 5-10 μm, our results suggest that O_3 was not capable of fully penetrating the NLF to reach epithelial cells. Application of Eq. 3.6, which is a simplification of Eq. 3.5 in the limit of infinite NLF thickness, yielded a

concentration profile that directly overlaps that of Fig. 5.4 for finite NLF thickness. This means that when the NLL thickness exceeds 0.6 μm, the concentration profile becomes independent of NLL thickness, implying that the system indeed behaves as an infinitely deep sink for O_3 uptake.

Because of substantial differences in the geometry and chemistry of the liquid lining layers in the nasal cavities and lung airways, direct extrapolation of our data may not accurately predict penetration depth in the lining layer of the lung. However, basic comparative predictions of effects of several parameters can be made by neglecting variations in geometry. Under this simplification, O_3 penetration depends on two factors. Firstly, there is a thinner lining layer in the distal airway of the lung than in the nose. Therefore the condition that O_3 concentration diminishes completely before reaching the cell wall may not hold. Secondly, antioxidant activity is higher in the lung lining fluid (Cross at al., 1994), with greater levels of ascorbic acid and glutathione, in addition to enzymatic antioxidants. Therefore, the penetration of O_3 in the lung lining layer depends on a balance between lung lining layer thickness and antioxidant composition.

5.7 Evaluation of Reaction-Diffusion Model Assumptions and Sensitivity Analysis

This section provides a discussion of factors and assumptions of the O_3 reaction-diffusion model (Section 3.4). A sensitivity analysis was performed to determine the effect of variation in estimated values of model parameters on the observed value of k_2 (Fig. 5.5).

At every sampling point, three consecutive measurements of Λ were taken. It was assumed that, throughout the 15 seconds of acquisition, the small dose of O_3 delivered to the nose from each measurement did not alter C_{UA} in the lining fluid. However, even short exposures may rapidly form a thin layer of depleted UA at the surface of the NLF, resulting in lowered Λ. This would result in measured values of Λ that are lower than predicted by the model, which assumes constant, evenly distributed uric acid in the NLF. Because values of $-\ln(1-\Lambda)$ are more sensitive to change at higher values of Λ, this phenomenon would give rise to a result where the observed slope of regression between $-\ln(1-\Lambda)$ versus $C_{UA}{}^{1/2}$ would be lower than expected, indicating a lower observed value for k_2. Therefore, apparent values of k_2 should be higher than observed in our study. However, our data suggests that development of a thin UA-depleted layer at the airway surface was not likely, as subsequent measurements of Λ were similar.

To relate the overall to tissue-phase mass transfer coefficients, we applied results obtained by Santiago et al. (2001), which determined relative contributions of gas and NLF phases to overall mass transfer, as a function of gas flow rate (See Eq. 3.11). Sensitivity analysis for 1-f shows that there is little effect of NLF phase resistance to mass transfer above 1-f=0.70 (Fig 5.5). This threshold is below the value of 0.77 used in our study at 3 lpm. Therefore, we conclude that variations in the relative contributions of gas and NLF phase resistances to mass transfer minimally influence estimates of k_2.

The surface area of the nasal cavity, S, used in this study was 270 cm^2 (Guilmette et al., 1989). However, there are inter-subject variations in nasal surface area. Overestimation of

S would artificially drive the apparent value of k_2 lower by attributing reactive uptake to a larger surface area. However, sensitivity analysis suggests that calculated values of k_2 do not change appreciably with values of S increased above 270 cm^2 (Fig 5.5). On the other hand, k_2 is strongly dependent on S below 200 cm^2. This may have been a factor if subjects experienced nasal congestion during the experiment, which could have effectively closed sections of the nasal cavity to gas flow, thus decreasing the effective value of S and leading to a deflated observed value for k_2. This is most probable in the turbinate region of the nose where the airways are rather thin. Finally, due to anatomical variations, some subjects' nasal cavities may include airspaces that restricted gas flow, such as dead spaces or areas of high resistance to flow.

We assumed the diffusion coefficient of O_3 in the mucus layer, D_{AB}, is the same as that in aqueous solution (Miller et al., 1985). Indeed, mucus is an aqueous solution with about 5% mucin, a large branched glycoprotein. On a molecular scale, the same cross linking that gives mucus a gel-like quality may hinder O_3 transport by diffusion, and lower D_{AB}. The assumed value for D_{AB} in this study was 2.7×10^{-5} cm^2/s. Sensitivity analysis demonstrates values for D_{AB} below our assumed value result in significant increases in corresponding values of k_2. Unfortunately, no experimental data is available on the diffusion of O_3 (or similar gases) through mucus. However, it is a well accepted assumption that small molecules have similar diffusion behavior in mucus and water.

Uric acid concentration in the NLF was determined by multiplying C_{UA} measured in nasal lavage by a dilution factor, d, obtained experimentally by Santiago et al. The value of d

was 11 M_{NLF}/M_{Lav}, where M_{NLF} and M_{LAV} represent uric acid concentration units in the NLF and nasal lavage samples, respectively. Values of d in Santiago's study ranged from 5-25 (2001). Van der Vliet (1999) reported values for d between 10-20. Cross et al (1994) assumed a value of 40. Sensitivity analysis shows a strong dependence of k_2 on d in the range of values reported (Fig. 5.5). Based on previous findings for values of d, it is most probable that d was underestimated in our study. Underestimation of d would attribute O_3 uptake to occurring via reaction with an estimated UA concentration that is less than that actually in the nasal lining layer. Thus, observed values of k_2 would be higher than the actual value.

In summary, the sensitivity analysis presented here suggests that, for each individual parameter (1-f, S, D_{AB}, or d) evaluated, the outcome for k_2 was within an order of magnitude of that observed in our study. It must be noted that this is only true for a certain range of values of the varied parameters, and does not take into account gross deviations from our assumed values.

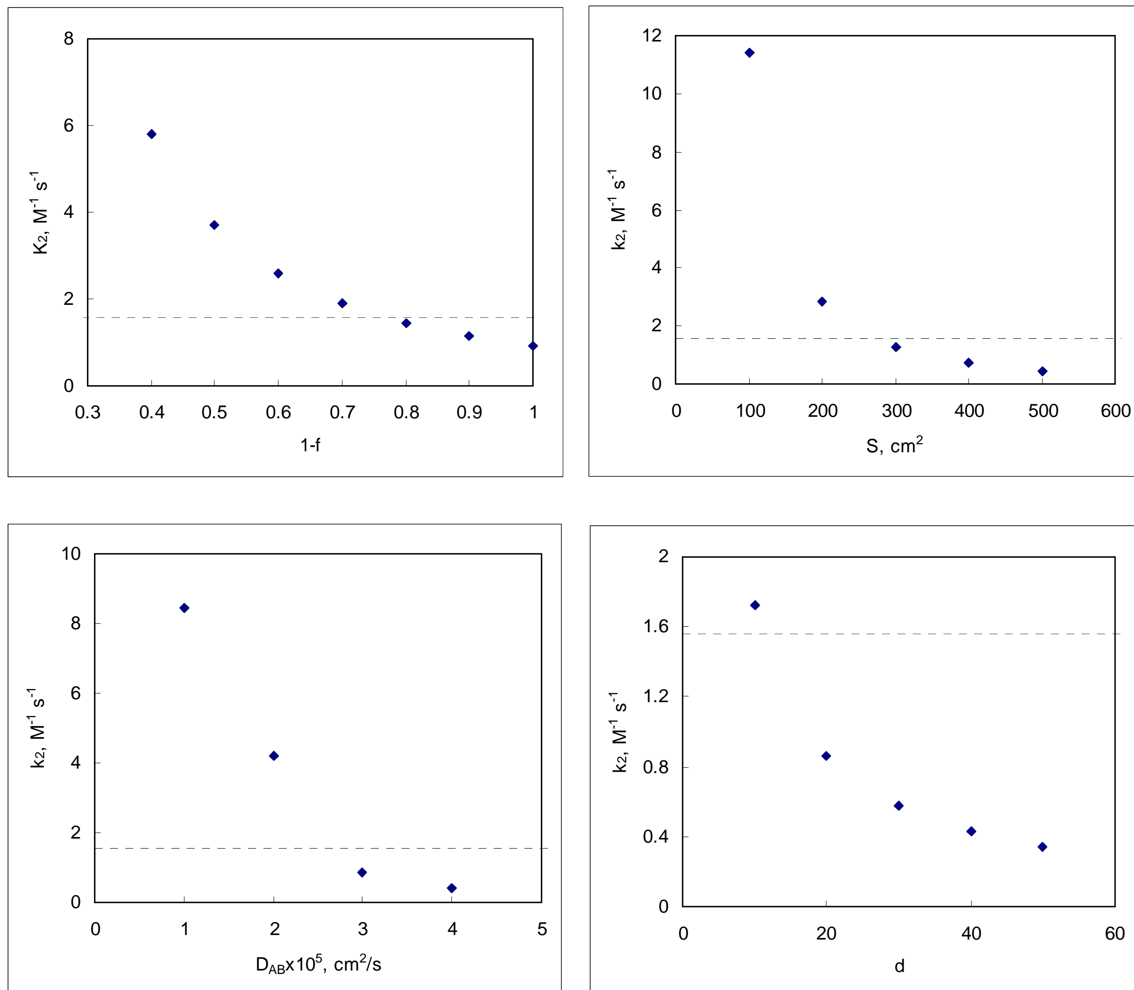


Figure 5.5 Sensitivity analysis of model parameters on the estimated value of k_2. Dashed lines indicate the value of k_2 obtained using the following parameter values: 1-f=0.77, S=270 cm^2, D_{AB}=2.7x10^5 cm^2/s, d=11.

5.8 The Case for Uric Acid as the Main Contributor to O_3 Uptake in the Nose

In this work, nasal uptake of O_3 was fully attributed to occurring via reaction with UA. The hypothesis that UA is the major antioxidant responsible for scavenging O_3 was based on findings by Peden et al (1990). This section summarizes results from our study that can be used to assess the validity of this assumption.

Firstly, the results of protocol AF2 showed that mean Λ and C_{UA} were both reduced following exposure to O_3, giving one indication that these parameters are related. Additionally, we observed that Λ was strongly dependent on C_{UA} within subject (Fig. 4.18). Application of the reaction-diffusion model indicated a very significant correlation, with a zero intercept for $-\ln(1-\Lambda)$, suggesting there would be no O_3 uptake in the absence of UA.

Studies employing nasal lavage (Protocol LS1) showed similar results. Mean Λ and C_{UA} exhibited a coordinated reduction following each subsequent nasal lavage maneuver. This result was also strongly observed on a intra-subject basis (Fig. D.2). However, application of the reaction-diffusion model indicated a significant positive intercept for $-\ln(1-\Lambda)$, most likely due to reaction between O_3 and exposed cell membrane constituents (Section 5.4). Although the relative contribution of cell membrane constituents was not assessed, the value for the k_2 arising from this

experiment was similar to that obtained from protocol AF2. The introduction of O_3-cell membrane interactions likely increased the measured value of Λ measured post lavage. Therefore, the observed slope of the relationship described by Eq 3.13 would be deflated, suggesting that k_2 obtained from protocol LS1 should be lower than that obtained from AF2, which is in agreement with our findings.

Because NO_2 is an oxidative compound, we expected it to oxidize NLF antioxidants, and subsequently lower Λ. However, NO_2 exposure (Protocol AF3) resulted in minimal reduction in Λ, and no change in C_{UA}. The reduction in Λ following NO_2 exposure must have been due to oxidation of compounds other than UA that are also susceptible to ozonation, such as ascorbic acid and proteins. Keeping in mind that there was only a minimal reduction in Λ, we can conclude that ozone uptake was not appreciably influenced by compounds that were oxidized by NO_2. Because UA is not one of these compounds, this finding is in line with our assumption that UA is the major scavenger of O_3 in the nose. This is similar to the result found by Santiago (2001) where methacholine challenge was used to induce mucus secretion in the nose. In their study, no changes in Λ or C_{UA} were observed, indicating that compounds released in response to methacholine were not appreciably susceptible to ozonation.

Chapter 6: Summary and Future Work

This main motivation for this study was to understand the reaction dependence of O_3 uptake in the nasal cavity. In particular, we aimed to determine the role of uric acid in modulating Λ. Based on previously reported results, it was hypothesized that reaction with UA is the major driver of O_3 from the airways into the NLF (Peden). The notable existence of day-to-day variations in system parameters hinders the observation of a relationship between Λ and C_{UA} on a day-to-day basis (Santiago et al., 2001). Therefore, several approaches were designed to impose changes in C_{UA} such that the relationship between Λ and C_{UA} can be determined within one experimental session.

In the pilot study (Protocol AF1), we showed that values of Λ were reduced following O_3 exposure. We hypothesized that this reduction in uptake was due to reactive depletion of uric acid by reaction with O_3. Therefore, in the following study (Protocol AF2), we investigated the relationship between Λ and UA concentrations in the NLF by measuring these parameters before and after O_3 exposure. We observed a coordinated lowering of values for Λ and C_{UA} following O_3 exposure. Application of a steady state one-dimensional reaction-diffusion model in the NLF, and a gas uptake model, allowed

us to relate O_3 uptake to system parameters in the NLF, including k_2, the second order reaction rate constant between O_3 and UA. Regression of this data yielded a significant result, with an apparent k_2=1.56x10^9±0.744x10^9 $M^{-1}s^{-1}$ arising from the slope. In support of our hypothesis that UA is the main target of O_3 oxidation, the regression exhibited a y-intercept that was not statistically different from zero, indicating that no uptake can occur in the absence of UA.

In another analysis, we analyzed Λ and C_{UA} data previously gathered from a perturbation study where contents of the nasal cavity were consecutively washed out once every minute for three minutes by nasal lavage (LS1). This resulted in successive reduction of Λ and C_{UA}, and significant correlation of these two variables. k_2 arising from the estimated slope of the regression was 6.04 x10^8±1.71 x10^8 $M^{-1}s^{-1}$, which is in rough agreement with the value of k_2 determined from the O_3 exposure study. One result arising from the washout study that was not observed in the O_3 exposure study was that the intercept of regression was significantly non-zero (Figure D.2), indicating that O_3 uptake can occur in the absence of UA. We postulate that this was due to exposure of the cell membrane following the repeated nasal lavage procedures. To verify this, future studies must investigate the contribution of cell membrane constituents to Λ. This can be done by monitoring the formation of membrane oxidation markers, such as acetylcholinesterase (Ballinger et al., 2005), following a series of nasal lavage maneuvers succeeded by O_3 exposure. The underlying assumption in such a study would be that repeated serial washout of the NLF would

directly expose cells to the gas phase. Immediate successive exposure to O_3 would facilitate direct membrane oxidation, marked by release of membrane-bound acetylcholinesterase.

In a third study (AF3), we exposed the nose to the oxidant gas NO_2. There was a small yet significant reduction of Λ following exposure, and no change in C_{UA}. This prompted us to investigate the reactivity between NO_2 and UA in model solutions. In support of our findings in vivo, we found that NO_2 shows little reactivity with UA compared to O_3. Considering that there was barely a change in Λ following exposure, we can safely conclude that our findings here do not conflict with the assumption that UA is the main contributor to O_3 uptake in the nose.

In this work, we determined k_2 by imposing nasal perturbations that reduce C_{UA} below basal values. In addition, we have applied in vivo data to provide evidence that UA is the major antioxidant driving O_3 absorption in the nose. This finding can be further validated in vivo by conducting similar studies that induce changes in NLF UA levels, such as 1) administration of nasal spray containing UA or uricase, 2) pharmacological intervention by xanthine oxidase inhibitors, or 3) stimulation of the gustatory reflex.

The concentration profile of O_3 in the NLF yielded identical results whenever the NLF thickness was equal to or greater than 1 μm. The penetration depth of O_3 into the NLF was estimated to be roughly 0.6 μm, which is markedly less than NLF thickness

estimates of 5-10 μm (Hatch, 1992), verifying that O_3 does not reach epithelial cells, and that toxic effects of O_3 must occur through secondary mechanisms. Our data can be applied to more geometrically accurate models of the nasal cavity to obtain values of k_2 in closer agreement with those obtained from in vitro solutions. This would allow more reliable estimation for O_3 penetration depth.

Another purpose of this work was to identify a physiologically relevant response marker to O_3 exposure. We showed that there was a small significant elevation of NO 1-h following initiation of exposure, indicating an emerging inflammatory presence. This is the first study in the literature to demonstrate increased NO levels in the human airways following controlled exposure to O_3. This may be a useful discovery for the development of NO as an easily-measured biomarker of the response to O_3 exposure. Knowing that the exposure conditions we used induced an NO response, future studies should aim at monitoring NO concentrations 6-8 hours following O_3 exposure, when inflammation is expected to peak. To amplify the response, studies may be designed to wash out antioxidant defenses by performing a series of nasal lavages prior to exposure, thereby increasing O_3-tissue interactions. Additionally, we showed increased TBARS levels in nasal lavage samples following O_3 exposure, indicating lipid peroxidation. Interestingly, it appeared that UA played a regulatory role in TBARS formation. An interesting study would be to determine if there is an O_3 dose dependence on TBARS and NO responses. Additionally, it would be exciting to further investigate the inhibitory role of UA on responses in these parameters during O_3 exposure. This may

ultimately lead to the development of a solution to overcome O_3 toxicity in sensitive populations, such as asthmatics.

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Appendix A: Human Subject Questionnaires

A.1. Medical History Screening Questionnaire

Date ______________________________

Name (Code)

____

Address

____

____

____

Phone (Business/Home) ______________________________

E-Mail ______________________________

Age (yrs) ______________________________

Height (in) ______________________________

Weight (lb) ______________________________

Sex ______________________________

Occupation ______________________________
(If a student, what major?)

SCREENING QUESTIONNAIRE(Continued)

Please answer the following questions to the best of your knowledge. For any questions marked "yes" please give details such as frequency of occurrence, severity, nature of diagnosis, type of medication, etc on page 4.

	Question	YES	NO
1.	Did you previously reside in a large city where air pollution may have been excessive?	☐	☐
2.	Have you smoked cigarettes, cigars or a pipe within the past 3 years (If you have, complete the Smoking Questionaire)?	☐	☐
3.	Do you exercise on a regular basis?	☐	☐
4.	Have you experienced respiratory symptoms such as chest discomfort or difficulty in breathing while exercising?	☐	☐
5.	Do you often walk, jog, bicycle, or motorcycle near motor vehicle traffic?	☐	☐
6.	Do you often suffer from sinus, nasal, or lung congestion?	☐	☐
7.	Do you experience seasonal Hay Fever-type symptoms such as watery, burning eyes; running nose; sneezing; and itchy throat?	☐	☐
8.	Do you experience chest pain, palpitations, dizziness, shortness of breath, or coughing spells?	☐	☐
9.	Have you ever been diagnosed or tested positively for a specific lung disease such as asthma, emphysema, chronic bronchitis, or tuberculosis?	☐	☐
10.	Have you ever had a heart attack or been diagnosed for high blood pressure, rapid heart rate, angina, or any other cardiovascular abnormality?	☐	☐
11.	Are you allergic to any drugs, or have you ever had an unexpected reaction to a drug?		

SCREENING QUESTIONNAIRE(Continued)

		YES	NO
12a.	Do you regularly take medication of any kind that requires a doctor's prescription? (For example, birth control pills or allergy medication)	☐	☐
12b	Do you regularly take any "over-the-counter" medications that do not require a doctor's perscription?	☐	☐
13.	Do you have <u>any</u> medical problems not mentioned in the above questions?	☐	☐
	14. For women subjects:		
	Is it possible that you are pregnant?	☐	☐
	Do you have a regular menstrual cycle?	☐	☐
	(If so, it is about _________ days long)		
	When did your last period begin?	About________days ago.	

A.2. Research Session Questionnaire

Session Number:
Date:
Subject Name (Code):

Purpose of Session:

1. Have you experienced a respiratory illness within the past two weeks?
   □ Yes □ No
   If so, briefly describe symptoms, treatment, and when the illness terminated:

2. Have you taken medication during the past two weeks ?
   □ Yes □ No
   If so, what was the medication and when was its use terminated:

3. For women:

   a. Is it possible that you are pregnant?
      □ Yes □ No

   b. Do you have a regular menstrual cycle?
      □ Yes □ No
      If so, it is about _______ days long.

   c. When did your last period begin ?
      About _________ days ago.

   d. I have been given a urine test for pregnancy, today, and I have been notified that this test indicates that I am not pregnant.
      □ Yes □ No

RESEARCH SESSION QUESTIONNAIRE (Continued)

Rate how you now feel with respect to these symptoms by circling the appropriate number:

0=none
1=just perceptible
2=distinctly perceptible
3=nuisance
4=offensive
5=unbearable

--

Symptom	Beginning of Session	End of Session
Headache	0 1 2 3 4 5	0 1 2 3 4 5
Nasal Discharge	0 1 2 3 4 5	0 1 2 3 4 5
Shortness of Breath	0 1 2 3 4 5	0 1 2 3 4 5
Cough or Urge to Cough	0 1 2 3 4 5	0 1 2 3 4 5
Chest Burning or Discomfort	0 1 2 3 4 5	0 1 2 3 4 5
Difficulty in Taking Deep Breath	0 1 2 3 4 5	0 1 2 3 4 5

____

Signature of Subject

A.3. Study Advertisement Flyer

Do you have a few hours to spare?

Earn Extra Cash

Are you a Healthy, Nonsmoker between the ages of 18 and 30?

Participate in an Ozone Study

Three 3-hour sessions

For More Information Contact Ali at 863-4027 or **email axf174@psu.edu**

A.4. Smoking History Assessment

Smoking Questionnaire

Noll Laboratory – Penn State University

Smoking History Questionnaire

Please circle the appropriate answer:

1. Do you smoke cigarettes at present? Yes No
(If not, skip to question 5)

2. If 'yes' how many packs per day?

Less than ½ pack ½ to 1 pack 1 to 2 packs more than 2 packs

3. Do you inhale the smoke? Yes No

4. How long have you been smoking?

Less than 1 year 1 to 5 years more than 5 years

5. Did you smoke cigarettes in the past year and quit permanently? Yes No
(If not, skip to question 9)

6. If 'yes' how many packs per day did you smoke?

Less than ½ pack ½ to 1 pack 1 to 2 packs more than 2 packs

7. Did you inhale? Yes No

8. When did you quit?

Less than 1 year 1 to 5 years more than 5 years

9. Do you smoke cigars at present? Yes No
(If not, skip to question 11)

10. If 'yes' how many cigars do you smoke per day?

Less than 2 2 to 5 more than 5

11. Did you smoke cigars in the past and quit permanently? Yes No

12. Do you smoke a pipe at present? Yes No
(If not, skip to question 14)

13. If 'yes' how many pipefulls do you smoke per day?

Less than 2 2 to 5 more than 5

14. Did you smoke a pipe in the past and quit permanently? Yes No

Appendix B: Approved Consent Forms for Clinical Studies

B.1. Protocol AF1

INFORMED CONSENT

Nasal Uptake of Ozone During Continuous Exposure

Title of Project: Ozone Exposure and Dose Delivered to Human Lungs
Subproject: Ozone Uptake and NO Production During Nasal Ozone Exposure (AF-1)
Principal Investigator: James S. Ultman, Ph.D.

Other Investigators: Ali Fassih, Aziz Ben-Jebria, Ph.D., Jan Ulbrecht, M.D.

Date: January 6th, 2003

This is to certify that I, , have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. James Ultman.

1. Purpose of the Study

Ozone is an irritant, pollutant gas that results from automobile exhaust. We are trying to understand how inhaled ozone is absorbed in the respiratory system and how the respiratory system responds to the absorbed ozone. We think that continuous breathing of ozone influences the removal of ozone by the nose. In particular, we expect that natural occurring protective compounds will be temporarily depleted so that ozone removal by the nose will decrease as ozone exposure progresses.

2. Procedures to be Followed

I understand that I will be taking part in a research project carried out in the General Clinical Research Center under the supervision of the medical staff of the Center. I realize that I will be checked in at the Nurses Station prior to each session and I should not leave the center until I am discharged by a nurse at the end of each session.

I understand that prior to my participation in the two research sessions of this study, I will be scheduled for a health screening session for obtaining medical information. I will sign this informed consent form, complete medical questionnaires, and be given a

routine medical examination including several breathing tests. I understand that I will be included in this study only if: I am a nonsmoker with no respiratory and nasal diseases and allergies or any other diseases; and I do not regularly take anti-histamines, decongestants, analgesics, or anti-inflammatory drugs. If I am a female subject, I understand that I will not be included in this study if I am pregnant. I understand that I must be at least 18 years of age to participate in this research project.

Following the health screening session, I understand that I will participate in two research sessions, each requiring about three hours to complete. I understand that during one of these research sessions, I will breath air containing a low concentration of ozone through my nose for 30 minutes and during the other research session I will breath "clean air" through my nose for 30 minutes. I realize that I will not be told when I am breathing clean air and when I am breathing ozonated air. During both research sessions, I agree to undergo several tests that measure lung function, nasal ozone removal and nasal nitric oxide production just before the 30-minute exposure to ozonated air or clean air begins and to repeat these measurements a few times within the hour after the exposure ends.
I understand that lung function will be measured by a standard pulmonary function test using an automated pulmonary function (PFT) instrument. In this test, I will be required to forcibly expire through a tube for about 7 seconds. My nasal volume will be measured by a standard acoustic rhinometry (AR) technique using a clinical instrument. In this test, I will be required to place a tube against each nostril while a sound pulse travels into my nasal cavity.

If I experience a respiratory illness or am required to temporarily take medication within one week before a session is scheduled, I will notify an investigator as soon as possible so the session can be rescheduled when I am feeling better. If I am a female subject, I agree to contribute a small urine specimen at the beginning of each session to be used in a three-minute pregnancy test. I understand that I should not participate in the study if these test results are positive, or if for any reason I believe that I am pregnant.

3. Discomforts and Risks

One of the risks in these experiments is the exposure of my nose and lungs to ozone. I understand that my nose will come in contact with air containing ozone and that I will inhale some of the ozone into my lungs. The level of ozone will be about the same as what I would inhale in a big city like Los Angeles or Houston on a smog alert day. But, I will only be breathing this ozone for 30 minutes.

I understand that even such limited exposures to ozone can irritate the respiratory system, causing coughing, shortness of breath, or discomfort in the nose or in the chest. These symptoms usually disappear within a short time following exposure. Even so, I understand that the long-term effects of ozone exposure are not known for sure.

If I should develop a symptom during a research session, I agree to remain in the laboratory until I am discharged by a medical staff member of the General Clinical

Research Center. If the medical staff feels it necessary for me to be treated in the Emergency Room at Center Community Hospital, I agree to do so. I further agree to return to the GCRC for a follow-up medical evaluation, if this is deemed necessary by the medical staff.

I realize that there is a possibility of intersubject transmission of infection from contact with the breathing equipment. I know that this risk will be minimized by using disposables when possible and by first cleaning in a soap solution followed by soaking in a medical disinfectant.

4. Benefits

a. Benefits to me. I understand that I will have access to the results of my medical examination and my breathing tests.

b. Benefits to society. This investigation will determine the relationship between ozone exposure and the dose retained in the nose. I understand that such data is used by the EPA as an aid in setting new regulatory standards for ozone.

5. Alternative Procedures

I understand that experiments could alternatively be carried out on laboratory animals. However, because the animals would have to be sedated and because of differences between animal and human physiology, the data obtained on animals would not be directly applicable to people. I realize that it is vital for these experiments be carried out on human subjects like myself.

6. Time Required

To complete a protocol, I agree to participate in a health screening session and two research sessions lasting about three hours each.

7. Confidentiality

I understand that all records associated with my participation in the study will be subject to the usual confidentiality standards applicable to medical records, and in the event of any publication resulting from the research no personally identifiable information will be disclosed.

8. Right to Ask Questions

I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction. If, at a later time, I have questions about the research or my participation in the research, I am aware that I should contact:

Dr. Aziz Ben-Jebria
863-8049 (Weekdays)

237-0739 (Evenings & Weekends)

In the event of a research-related injury, I realize that I should contact:

Dr. James Ultman
863-4802 (Weekdays)
237-6335 (Evenings & Weekends)

For questions regarding my rights as a research participant I will contact the Office for Research Protections at (814) 865-1775. This office is located at 212 Kern Building, University Park, PA 16802.

9. Compensation

I understand that I will be compensated at a rate of $10.00 per hour for the sessions in which I participate. If I am an employee of Penn State University, the compensation I receive for participation will be treated as taxable income, and therefore taxes will be taken from the total amount. If I am not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require me to claim the compensation that I receive for participation in this study as taxable income

10. Voluntary Participation.

I understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying any of the investigators.

11. In the event that abnormal test results are obtained, I understand that I will be apprised of the results and recommended to contact my private medical provider for follow-up.

12. I understand that medical care is available in the event of injury resulting from research but that neither financial compensation nor free medical treatment is provided. I also understand that I am not waiving any rights that I may have against the University for injury resulting from negligence of the University or investigators.

This is to certify that I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

____

Volunteer Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

.____

Investigator Date

B.2. Protocol AF2

INFORMED CONSENT
Nasal Uptake of Ozone During Continuous Exposure

Title of Project: Ozone Exposure and Dose Delivered to Human Lungs
Subproject: Ozone Uptake and NO Production During Nasal Ozone Exposure (AF-1)
Principal Investigator: James S. Ultman, Ph.D.
Other Investigators: Ali Fassih, Aziz Ben-Jebria, Ph.D., Felix Meza, M.D., Melissa Lowe

This is to certify that I, ____________________, have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. James Ultman.

1. Purpose of the Study

Ozone is an irritant, pollutant gas that results from automobile exhaust. We are trying to understand how inhaled ozone is absorbed in the respiratory system and how the respiratory system responds to the absorbed ozone. We think that continuous breathing of ozone influences the removal of ozone by the nose. In particular, we expect that natural occurring protective compounds will be temporarily depleted so that ozone removal by the nose will decrease as ozone exposure progresses.

2. Procedures to be Followed

I understand that I will be taking part in a research project carried out in the General Clinical Research Center (GCRC) under the supervision of the medical staff of the Center. I realize that I will be checked in at the Nurses Station prior to each session and I should not leave the center until I am discharged by a nurse at the end of each session.

I understand that prior to my participation in the two research sessions of this study, I will be scheduled for a health screening session for obtaining medical information. I will sign this informed consent form, complete medical questionnaires, and be given a routine medical examination including several breathing tests. I understand that I will be included in this study only if: I am a nonsmoker with no respiratory and nasal diseases and

allergies or any other diseases; and I do not regularly take anti-histamines, decongestants, analgesics, or anti-inflammatory drugs. If I am a female subject, I understand that I will not be included in this study if I am pregnant. I understand that I must be at least 18 years of age to participate in this research project.

Following the health screening session, I understand that I will participate in two research sessions, each requiring about three hours to complete. I understand that during one of these research sessions, I will breath air containing a low concentration of ozone through my nose for 30 minutes and during the other research session I will breath "clean air" through my nose for 30 minutes. I realize that I will not be told when I am breathing clean air and when I am breathing ozonated air. During both research sessions, I agree to undergo several tests that measure lung function, nasal ozone removal and nasal nitric oxide production just before the 30-minute exposure to ozonated air or clean air begins and to repeat these measurements a few times within the hour after the exposure ends. I understand that lung function will be measured by a standard pulmonary function test using an automated pulmonary function (PFT) instrument. In this test, I will be required to forcibly expire through a tube for about 7 seconds. Nasal ozone removal will be measured by holding two tubes against my nostrils and allowing a humidified air sample with a low concentration of ozone to circulate through my nose for 15 seconds. During this procedure, I understand that I will be forcibly exhaling through my mouth in order to isolate my nose from my mouth. This will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement. Nasal nitric oxide production will be measured by holding two tubes against my nostrils while clean humidified air circulates through my nose. This will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement. My nasal volume will be measured by a standard acoustic rhinometry (AR) technique using a clinical instrument. In this test, I will be required to place a tube against each nostril while a sound pulse travels into my nasal cavity. This will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement. I also understand that the contents of my nasal cavity will be sampled by performing washout with about two teaspoons of mild salt solution. This will be done by holding my breath and tilting my head back while the salt solution is introduced into my nose. I will hold my breath in order to avoid leakage into my throat. Ten seconds later, I will tilt my head forward and release the contents into a specimen cup. This will be immediately repeated for the opposite nostril. This will be done once at the beginning of the experiment, and once again at 90 and 150 minutes into the experiment. After the sessions, samples obtained will be analyzed for antioxidants, nitrite, protein, and lipid peroxidation markers.

If I experience a respiratory illness or am required to temporarily take medication within one week before a session is scheduled, I will notify an investigator as soon as possible so the session can be rescheduled when I am feeling better. If I am a female

subject, I agree to contribute a small urine specimen at the beginning of each session to be used in a three-minute pregnancy test. I understand that I should not participate in the study if these test results are positive, or if for any reason I believe that I am pregnant.

3. Discomforts and Risks

One of the risks in these experiments is the exposure of my nose and lungs to ozone. I understand that my nose will come in contact with air containing ozone and that I will inhale some of the ozone into my lungs. The level of ozone will be about the same as what I would inhale in a big city like Los Angeles or Houston on a smog alert day. But, I will only be breathing this ozone for 30 minutes.

I understand that even such limited exposures to ozone can irritate the respiratory system, causing coughing, shortness of breath, or discomfort in the nose or in the chest. These symptoms usually disappear within a short time following exposure. Even so, I understand that the long-term effects of ozone exposure are not known for sure.

If I should develop a symptom during a research session, I agree to remain in the laboratory until I am discharged by a medical staff member of the General Clinical Research Center (GCRC). If the medical staff feels it necessary for me to be treated in the Emergency Room at Mount Nittany Medical Center, I agree to do so. I further agree to return to the GCRC for a follow-up medical evaluation, if this is deemed necessary by the medical staff.

I realize that there is a possibility of intersubject transmission of infection from contact with the breathing equipment. I know that this risk will be minimized by using disposables when possible and by first cleaning in a soap solution followed by soaking in a medical disinfectant. During nasal lavage, holding my breath will minimize the risk of saline dripping into my throat and causing cough. A possible risk of infection from nasal lavage will be minimized by using sterile dispensing tubes and sterile salt solution.

4. Benefits

a. Benefits to me. I understand that I will have access to the results of my medical examination and my breathing tests.

b. Benefits to society. This investigation will determine the relationship between ozone exposure and the dose retained in the nose. I understand that such data is used by the EPA as an aid in setting new regulatory standards for ozone.

5. Alternative Procedures

I understand that experiments could alternatively be carried out on laboratory animals. However, because the animals would have to be sedated and because of differences between animal and human physiology, the data obtained on animals would not be directly applicable to people. I realize that it is vital for these experiments be carried out on human subjects like myself.

6. Time Required

To complete a protocol, I agree to participate in a health screening session and two research sessions lasting about three hours each.

7. Confidentiality

I understand that all records associated with my participation in the study will be subject to the usual confidentiality standards applicable to medical records, and in the event of any publication resulting from the research no personally identifiable information will be disclosed. The Office for Research Protections and the Biomedical Institutional Review Board (IRB) may review records related to this project.

8. Right to Ask Questions

I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction. If, at a later time, I have questions about the research or my participation in the research, I am aware that I should contact:

Dr. Aziz Ben-Jebria
863-8049 (Weekdays)
237-0739 (Evenings & Weekends)

In the event of a research-related injury, I realize that I should contact:

Dr. James Ultman
863-4802 (Weekdays)
237-6335 (Evenings & Weekends)

For questions regarding my rights as a research participant I will contact the Office for Research Protections at (814) 865-1775. This office is located at 212 Kern Building, University Park, PA 16802.

9. Compensation

I understand that I will be compensated at a rate of $10.00 per hour for the sessions in which I participate. If I am an employee of Penn State University, the compensation I receive for participation will be treated as taxable income, and therefore taxes will be taken from the total amount. If I am not employed by Penn State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require me to claim the compensation that I receive for participation in this study as taxable income

10. Voluntary Participation.

I understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying any of the investigators.

11. In the event that abnormal test results are obtained, I understand that I will be apprised of the results and recommended to contact my private medical provider for follow-up.

13. I understand that medical care is available in the event of injury resulting from research but that neither financial compensation nor free medical treatment is provided. I also understand that I am not waiving any rights that I may have against the University for injury resulting from negligence of the University or investigators.

This is to certify that I consent to and give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

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Volunteer Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

____

Investigator Date

B.2. Protocol AF3

INFORMED CONSENT FORM FORCLINICAL RESEARCH STUDY

The Pennsylvania State University

Title of Project: Ozone Exposure and Dose Delivered to Human Lungs
Principal Investigator: James S. Ultman, Ph.D.
Other Investigators: Ali Fassih, Aziz Ben-Jebria, Ph.D., Felix Meza, M.D., Melissa Lowe

This is to certify that I, ______________, have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. James Ultman.

1. Purpose of the Study

Ozone is an irritant, pollutant gas that results from automobile exhaust. We are trying to understand how inhaled ozone is absorbed in the respiratory system and how the respiratory system responds to the absorbed ozone. We think that continuous breathing of ozone influences the removal of ozone by the nose. In particular, we expect that natural occurring protective compounds such as antioxidants will be temporarily depleted so that ozone removal by the nose will decrease as ozone exposure progresses.

We are also interested in learning how exposure to other common pollutants can influence subsequent removal of ozone from the nose. In particular we wish to determine effects of nitrogen dioxide, and sulfur dioxide, two important gaseous constituents of polluted air in addition to ozone. The major source for nitric oxide in the atmosphere is combustion of fossil fuels for power generation, for heating, and in motor vehicles. Sources for sulfur dioxide include coal-fired electric power plants, wood pulp manufacturing, and food processing.

2. Procedures to be Followed

I understand that I will be taking part in a research project carried out in the General Clinical Research Center (GCRC) under the supervision of the medical staff of the Center. I realize that I will be checked in at the Nurses Station prior to each session and I should not leave the center until I am discharged by a nurse at the end of each session.

I understand that prior to my participation in the four research sessions of this study, I will be scheduled for a health screening session for obtaining medical information. I will sign this informed consent form, complete medical questionnaires, and be given a routine medical examination including several breathing tests. I understand that I will be included in this study only if: I am a nonsmoker with no respiratory or nasal diseases, allergies or any other diseases; and I do not regularly take anti-histamines, decongestants, analgesics, or anti-inflammatory drugs. If I am a female subject, I understand that I will not be included in this study if I am pregnant. I understand that I must be at least 18 years of age to participate in this research project.

Following the health screening session, I understand that I will participate in up to four research sessions, each requiring about three hours to complete. I understand that I will breathe either pure air or air containing a low concentration of ozone, nitrogen dioxide or sulfur dioxide through my nose for 30 minutes. I realize that I will not be told which stream I am inhaling. During each research sessions, I agree to undergo several tests that measure lung function, nasal ozone removal and nasal nitric oxide production just before the 30-minute exposure begins, and to repeat these measurements a few times within the hour after the exposure ends.

I understand that lung function will be measured by a standard pulmonary function test using an automated pulmonary function (PFT) instrument. In this test, I will be required to forcibly expire through a tube for about 7 seconds.

Nasal ozone removal will be measured by holding two tubes against my nostrils and allowing a humidified air sample with a low concentration of ozone to circulate through my nose for 15 seconds. During this procedure, I understand that I will be forcibly exhaling through my mouth in order to isolate my nose from my mouth. This

will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement.

Nasal nitric oxide production will be measured by holding two tubes against my nostrils while clean humidified air circulates through my nose. This will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement.

My nasal volume will be measured by a standard acoustic rhinometry (AR) technique using a clinical instrument. In this test, I will be required to place a tube against each nostril while a sound pulse travels into my nasal cavity. This will be done at the beginning of the experiment as well as 60, 90, 105, 120, and 150 minutes after the initial measurement.

I also understand that the contents of my nasal cavity will be sampled by performing washout with about two teaspoons of mild salt solution. This will be done by holding my breath and tilting my head back while the salt solution is introduced into my nose. I will hold my breath in order to avoid leakage into my throat. Ten seconds later, I will tilt my head forward and release the contents into a specimen cup. This will be immediately repeated for the opposite nostril. This will be done once at the beginning of the experiment, and once again at 90 and 150 minutes into the experiment. After the sessions, samples obtained will be analyzed for antioxidants, nitrite, protein, and lipid peroxidation markers.

If I experience a respiratory illness or am required to temporarily take medication within one week before a session is scheduled, I will notify an investigator as soon as possible so the session can be rescheduled when I am feeling better. If I am a female subject, I agree to contribute a small urine specimen at the beginning of each session to be used in a three-minute pregnancy test. I understand that I should not participate in the study if these test results are positive, or if for any reason I believe that I am pregnant.

3. Discomforts and Risks

One of the risks in these experiments is the exposure of my nose and lungs to ozone, nitrogen dioxide, and sulfur dioxide. I understand that my nose will come in contact with air containing these gases and that I will inhale some into my lungs. The level of ozone will be about the same as what I would inhale in a big city like Los Angeles or Houston on a smog alert day. The levels of nitrogen dioxide and sulfur dioxide will be similar to what can be encountered by workers in during their entire shift in a paper mill or in a electric power plant. But, I will only be breathing these mixtures for only 30 minutes.

I understand that even such limited exposures to ozone, nitrogen dioxide, and sulfur dioxide can irritate the respiratory system, causing headache, dizziness, coughing,

shortness of breath, or discomfort in the nose or in the chest. I understand that the levels I will be breathing are a fraction of the maximum 8-hour time-weighted averages prescribed by the Occupational Safety and Health Administration. I understand that the long-term effects of these gases are not known for sure.

If I should develop a symptom during a research session, I agree to remain in the laboratory until I am discharged by a medical staff member of the General Clinical Research Center (GCRC). If the medical staff feels it necessary for me to be treated in the Emergency Room at Mount Nittany Medical Center, I agree to do so. I further agree to return to the GCRC for a follow-up medical evaluation, if this is deemed necessary by the medical staff.

I realize that there is a possibility of intersubject transmission of infection from contact with the breathing equipment. I know that this risk will be minimized by using disposables when possible and by first cleaning in a soap solution followed by soaking in a medical disinfectant. During nasal lavage, holding my breath will minimize the risk of saline dripping into my throat and causing cough. A possible risk of infection from nasal lavage will be minimized by using sterile dispensing tubes and sterile salt solution.

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5. Alternative Procedures

I understand that experiments could alternatively be carried out on laboratory animals. However, because the animals would have to be sedated and because of differences between animal and human physiology, the data obtained on animals would not be directly applicable to people. I realize that it is vital for these experiments be carried out on human subjects like myself.

6. Time Required

To complete a protocol, I agree to participate in a health screening session and four research sessions lasting about three hours each.

7. Confidentiality

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11. In the event that abnormal test results are obtained, I understand that I will be apprised of the results and recommended to contact my private medical provider for follow-up.

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____

Volunteer Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

____

Investigator Date

Appendix C: Subject Data

C.1. AF1 Subject Data

C.1.1. Λ throughout air exposure sessions.

	Λ				
Subject	pre-exposure	post-exposure	15-post	30-post	60-post
AF1-01	0.743	0.829	0.809	0.798	0.773
AF1-02	0.737	0.774	0.775	0.797	0.786
AF1-03	0.864	0.871	0.807	0.865	0.824
AF1-04	0.775	0.807	0.744	0.753	0.750
AF1-05	0.896	0.888	0.846	0.855	0.928
AF1-06	0.859	0.859	0.804	0.847	0.844
AF1-07	0.713	0.744	0.722	0.688	0.727
AF1-08	0.743	0.787	0.821	0.821	0.867
AF1-09	0.923	0.855	0.915	0.923	0.903
AF1-10	0.797	0.797	0.785	0.839	0.851
AF1-11	0.671	0.612	0.604	0.613	0.630
AF1-12	0.809	0.820	0.812	0.807	0.805
AF1-13	0.874	0.893	0.855	0.863	0.851
AF1-14	0.922	0.983	0.967	0.890	0.886
AF1-15	0.886	0.928	0.964	0.961	0.943
Mean	**0.814**	**0.830**	**0.815**	**0.821**	**0.825**
SE	**0.021**	**0.022**	**0.024**	**0.023**	**0.021**

C.1.2. C_{NO} throughout air exposure sessions.

	C_{NO}, ppb				
Subject	pre-exposure	post-exposure	15-post	30-post	60-post
AF1-01	100.4	104.6	107.4	107.0	111.0
AF1-02	98.4	204.0	220.0	215.7	211.0
AF1-03					
AF1-04	109.9	124.7	109.5	118.2	117.2
AF1-05	101.4	97.4	101.5	97.3	95.6
AF1-06	134.9	118.0	127.4	148.6	140.1
AF1-07	157.7	170.8	160.0	146.9	144.8
AF1-08	131.0	140.8	152.8	154.6	157.8
AF1-09	61.4	82.9	81.5	87.3	80.5
AF1-10	87.8	93.5	85.4	95.0	90.4
AF1-11	75.9	76.2	74.1	74.7	78.3
AF1-12	90.3	91.4	85.7	90.0	92.1
AF1-13	69.9	79.8	74.4	77.2	77.5
AF1-14	128.6	158.6	145.0	140.5	140.5
AF1-15	152.6	142.8	178.3	146.9	154.8
Mean	**105.9**	**111.7**	**110.8**	**110.8**	**110.2**
SE	**9.1**	**9.1**	**10.0**	**8.2**	**8.2**

C.1.3. Nasal Volume throughout air exposure sessions

	NV, (cm^3)		
Subject	pre-exposure	post-exposure	60-post
AF1-01	53.1	48.4	45.5
AF1-02	51.9	50.7	50.2
AF1-03	57.6	50.3	61.3
AF1-04	96.5	77.9	67.7
AF1-05	37.9	49.2	57.7
AF1-06	73.5	44.2	52.3
AF1-07	54.0	54.4	57.2
AF1-08			
AF1-09	47.2	50.3	50.8
AF1-10	44.3	38.8	45.9
AF1-11	33.9	26.7	
AF1-12	48.1	40.2	45.9
AF1-13	65.0	56.0	55.6
AF1-14	55.2	48.0	52.4
AF1-15	68.2	50.1	52.5
Mean	**56.2**	**48.9**	**53.4**
SE	**4.1**	**2.9**	**1.7**

C.1.4. Λ throughout O_3 exposure sessions

	Λ				
Subject	pre-exposure	post-exposure	15-post	30-post	60-post
AF1-01	0.746	0.681	0.766	0.786	0.808
AF1-02	0.793	0.688	0.807	0.805	0.812
AF1-03	0.884	0.706	0.811	0.768	0.891
AF1-04	0.790	0.710	0.749	0.783	0.761
AF1-05	0.827	0.717	0.792	0.762	0.829
AF1-06	0.892	0.775	0.882	0.888	0.891
AF1-07	0.751	0.618	0.730	0.739	0.749
AF1-08	0.782	0.733	0.816	0.820	0.793
AF1-09	0.854	0.766	0.857	0.911	0.872
AF1-10	0.859	0.698	0.841	0.838	0.853
AF1-11	0.671	0.491	0.622	0.635	0.644
AF1-12	0.771	0.707	0.782	0.793	0.816
AF1-13	0.932	0.717	0.876	0.887	0.905
AF1-14	0.916	0.824	0.856	0.811	0.839
AF1-15	0.902	0.843	0.908	0.898	0.889
Mean	**0.825**	**0.712**	**0.806**	**0.808**	**0.823**
SE	**0.019**	**0.021**	**0.019**	**0.019**	**0.018**

C.1.5. C_{NO} throughout O_3 exposure sessions

	$C_{NO,}$(ppb)				
Subject	pre-exposure	post-exposure	15-post	30-post	60-post
AF1-01	102.8	122.3	137.5	129.9	127.5
AF1-02	126.9	134.6	130.4	131.8	127.6
AF1-03					
AF1-04	118.6	138.7	122.2	130.3	138.7
AF1-05	83.9	92.4	101.3	86.8	91.2
AF1-06	120.9	118.8	118.7	134.9	
AF1-07	169.8	169.7	176.2	169.7	165.2
AF1-08	200.4	159.1	157.1	138.5	138.2
AF1-09	70.1	80.4	81.9	82.2	75.3
AF1-10	82.7	100.6	89.4	97.2	99.4
AF1-11	84.3	90.3	93.5	84.5	88.9
AF1-12	66.3	64.4	74.5	78.4	72.0
AF1-13	87.2	72.8	72.9	83.7	84.9
AF1-14	110.7	102.3	97.3	100.2	103.9
AF1-15	135.3	142.2	143.5	148.6	148.6
Mean	**102.7**	**107.9**	**109.1**	**110.5**	**108.7**
SE	**8.7**	**9.0**	**9.1**	**8.9**	**9.1**

C.1.6. Nasal Volume throughout O_3 exposure sessions

	NV, (cm^3)		
Subject	pre-exposure	post-exposure	60-post
AF1-01	58.3	60.3	57.7
AF1-02	51.2	54.6	
AF1-03			
AF1-04	72.4	50.9	52.7
AF1-05	48.7	44.9	49.7
AF1-06	49.3	50.9	51.5
AF1-07	56.8	52.2	55.5
AF1-08	81.4	53.1	46.0
AF1-09	48.4	53.1	65.0
AF1-10	45.0	44.0	45.1
AF1-11	29.9	34.4	31.7
AF1-12	39.9	37.0	41.2
AF1-13	58.4	46.1	47.7
AF1-14	56.6	51.6	44.5
AF1-15	87.3	43.9	48.5
Mean	**55.9**	**48.3**	**49.0**
SE	**4.0**	**1.8**	**2.1**

C.2. AF2 Subject Data

C.2.1. Λ throughout air exposure sessions

	Λ					
Subject	60-pre	pre-exposure	post-exposure	15-post	30-post	60-post
AF2-01	0.752	0.832	0.777	0.799	0.844	0.836
AF2-02	0.836	0.810	0.822	0.795	0.818	0.862
AF2-03	0.844	0.780	0.757	0.675	0.698	0.792
AF2-04	0.610	0.630	0.629	0.599	0.600	0.646
AF2-05	0.970	0.979	0.976	0.933	0.951	0.984
AF2-06	0.853	0.885	0.878	0.877	0.868	0.839
AF2-07	0.675	0.643	0.709	0.669	0.666	0.689
AF2-08	0.680	0.641	0.700	0.678	0.725	0.724
AF2-09	0.801	0.876	0.877	0.771	0.914	0.921
AF2-10	0.779	0.796	0.801	0.780	0.781	0.809
AF2-11	0.772	0.798	0.779	0.730	0.724	0.739
AF3-01	0.730	0.789	0.848	0.828	0.812	0.780
AF3-02	0.874	0.867	0.854	0.808	0.802	0.820
AF3-03	0.794	0.901	0.909	0.865	0.838	0.830
AF3-04	0.648	0.628	0.627	0.587	0.590	0.619
AF3-05	0.751	0.810	0.856	0.810	0.796	0.806
AF3-06	0.814	0.800	0.809	0.849	0.819	0.842
AF3-07	0.723	0.760	0.769	0.745	0.751	0.769
AF3-08	0.833	0.812	0.856	0.856	0.814	0.811
AF3-09	0.738	0.748	0.792	0.780	0.782	0.732
AF3-10	0.719	0.744	0.652	0.728	0.725	0.755
AF3-11	0.734	0.815	0.816	0.812	0.812	0.824
AF3-12	0.789	0.790	0.668	0.731	0.757	0.731
AF3-13	0.793	0.807	0.839	0.769	0.773	0.815
AF3-14	0.934	0.920	0.921	0.909	0.915	0.921
Mean	**0.778**	**0.794**	**0.797**	**0.775**	**0.783**	**0.796**
SE	**0.017**	**0.018**	**0.018**	**0.018**	**0.018**	**0.017**

C.2.2. C_{UA} throughout air exposure sessions

	C_{UA}, (μM)		
Subject	60-pre	post-exposure	60-post
AF2-01	34.9	33.0	26.0
AF2-02	40.2	42.6	40.3
AF2-03	5.3	3.6	1.7
AF2-04	12.0	9.7	4.6
AF2-05	39.8	43.3	44.2
AF2-06	20.4	27.3	19.8
AF2-07	19.5	24.7	45.0
AF2-08	77.4	44.0	24.8
AF2-09	13.7	18.8	10.8
AF2-10	15.3	20.9	11.6
AF2-11	14.4	14.3	12.7
AF3-01	22.9	24.8	32.5
AF3-02	41.3	30.9	26.7
AF3-03	7.3	8.8	9.3
AF3-04	7.0	8.0	6.6
AF3-05	11.8	21.5	12.0
AF3-06	14.1	23.1	19.8
AF3-07	13.3	16.0	13.5
AF3-08	14.8	25.5	21.7
AF3-09	25.6	25.4	8.8
AF3-10	15.5	9.2	14.0
AF3-11	11.6	12.1	7.7
AF3-12	13.7	18.5	18.7
AF3-13	8.8	15.9	13.8
AF3-14	20.2	22.1	13.2
Mean	**20.8**	**21.8**	**18.4**
SE	**3.1**	**2.2**	**2.4**

C.2.3. Total Protein throughout air exposure sessions

	Total Protein, (μg,ml)		
Subject	60-pre	post-exposure	60-post
AF2-01	168.0	225.5	191.9
AF2-02	243.7	259.5	274.1
AF2-03	195.1	215.4	239.3
AF2-04	192.3	194.3	156.3
AF2-05	235.2	204.4	229.9
AF2-06	204.4	190.7	204.0
AF2-07	217.0	295.9	246.9
AF2-08	283.8	207.3	193.9
AF2-09	173.7	191.5	176.1
AF2-10	183.4	276.5	168.0
AF2-11	261.5	315.0	244.5
AF3-01	305.8	350.5	264.1
AF3-02			
AF3-03	117.2	140.1	193.7
AF3-04	266.5	134.9	125.5
AF3-05	272.8	150.9	123.8
AF3-06	179.5	235.3	238.8
AF3-07	208.5	303.2	126.3
AF3-08	163.9	119.7	115.6
AF3-09	315.5	245.8	172.8
AF3-10	122.2	109.0	102.3
AF3-11	83.5	128.0	134.9
AF3-12	504.8	408.1	326.9
AF3-13	361.1	283.1	259.1
AF3-14	171.6	81.3	297.7
Mean	**226.3**	**219.4**	**200.3**
SE	**18.2**	**16.8**	**12.7**

C.2.4. Λ throughout O_3 exposure sessions

	Λ					
Subject	60-pre	pre-exposure	post-exposure	15-post	30-post	60-post
AF2-01	0.756	0.833	0.622	0.819	0.824	0.815
AF2-02	0.800	0.814	0.670	0.826	0.819	0.869
AF2-03	0.829	0.821	0.715	0.786	0.893	
AF2-04	0.675	0.677	0.579	0.679	0.722	0.728
AF2-05	0.886	0.925	0.773	0.901	0.965	0.969
AF2-06	0.809	0.888	0.831	0.916	0.865	0.870
AF2-07	0.759	0.747	0.721	0.788	0.793	0.825
AF2-08	0.573	0.578	0.471	0.531	0.512	0.604
AF2-09	0.856	0.903	0.549	0.761	0.844	0.816
AF2-10	0.773	0.810	0.704	0.775	0.788	0.798
AF2-11	0.717	0.714	0.495	0.622	0.651	0.697
AF3-01	0.776	0.811	0.645	0.782	0.816	0.836
AF3-02						
AF3-03	0.834	0.890	0.703	0.887	0.891	0.913
AF3-04	0.703	0.667	0.504	0.605	0.631	0.574
AF3-05	0.734	0.847	0.728	0.762	0.792	0.801
AF3-06	0.904	0.994	0.650	0.944	0.942	0.865
AF3-07	0.664	0.705	0.579	0.676	0.723	0.709
AF3-08	0.878	0.852	0.724	0.889	0.875	0.897
AF3-09	0.728	0.712	0.644	0.735	0.718	0.734
AF3-10	0.712	0.770	0.597	0.687	0.726	0.710
AF3-11	0.760	0.810	0.752	0.832	0.866	0.842
AF3-12	0.725	0.824	0.660	0.771	0.817	0.815
AF3-13	0.789	0.830	0.644	0.798	0.819	0.807
AF3-14	0.932	0.747	0.596	0.755	0.772	0.768
Mean	**0.774**	**0.799**	**0.648**	**0.772**	**0.794**	**0.794**
SE	**0.017**	**0.019**	**0.019**	**0.021**	**0.021**	**0.019**

C.2.5 C_{UA} throughout O_3 exposure sessions

	C_{UA}, (μM)		
Subject	60-pre	post-exposure	60-post
AF2-01	31.1	29.9	29.0
AF2-02	48.2	40.8	41.9
AF2-03	5.0	5.5	2.9
AF2-04	20.4	8.3	6.5
AF2-05	31.1	20.4	20.1
AF2-06	18.4	17.4	22.2
AF2-07	34.8	36.7	56.4
AF2-08	13.3	10.8	14.5
AF2-09	18.9	13.0	15.5
AF2-10	12.2	8.2	11.2
AF2-11	20.1	3.1	9.0
AF3-01	32.4	22.6	26.5
AF3-02			
AF3-03	7.3	5.0	12.5
AF3-04	7.0	4.8	7.1
AF3-05	19.1	8.9	24.4
AF3-06	15.4	9.1	12.6
AF3-07	16.3	7.0	16.3
AF3-08	16.4	10.7	14.4
AF3-09	18.2	11.8	8.5
AF3-10	14.8	7.4	18.3
AF3-11	11.0	11.5	13.7
AF3-12	13.6	10.2	13.5
AF3-13	26.4	14.3	13.3
AF3-14	9.0	4.3	9.7
Mean	**19.2**	**13.4**	**17.5**
SE	**2.1**	**2.0**	**2.4**

C.2.6. Total Protein levels throughout O_3 exposure sessions

	Total Protein, (µg,ml)		
Subject	60-pre	post-exposure	60-post
AF2-01	256.3	264.0	234.4
AF2-02	272.9	307.7	234.0
AF2-03	223.9	227.5	226.3
AF2-04	221.4	234.4	235.2
AF2-05	209.3	236.0	236.4
AF2-06	178.1	181.4	174.5
AF2-07	205.7	199.2	205.7
AF2-08	266.4	238.4	234.8
AF2-09	228.3	250.2	218.6
AF2-10	165.2	160.7	172.1
AF2-11	278.9	183.4	187.4
AF3-01	245.1	285.3	161.1
AF3-02			
AF3-03	178.3	118.8	124.2
AF3-04	199.8	283.8	217.2
AF3-05	195.4	188.6	131.6
AF3-06	230.5	218.2	153.7
AF3-07	214.2	287.2	405.1
AF3-08	200.7	148.3	169.6
AF3-09	312.6	324.7	189.6
AF3-10	143.6	150.9	217.2
AF3-11	121.5	165.8	127.9
AF3-12	301.3	296.6	425.0
AF3-13	511.5	529.5	232.5
AF3-14	57.4	89.5	86.8
Mean	**225.8**	**232.1**	**208.4**
SE	**17.0**	**18.1**	**15.6**

C.3. AF3 Subject Data

C.3.1. Λ throughout NO_2 exposure sessions

	Λ					
Subject	60-pre	pre-exposure	post-exposure	15-post	30-post	60-post
AF3-01	0.830	0.852	0.836	0.838	0.850	0.840
AF3-02						
AF3-03	0.826	0.933	0.831	0.887	0.899	0.899
AF3-04	0.654	0.622	0.551	0.629	0.653	0.643
AF3-05	0.750	0.800	0.838	0.784	0.786	0.713
AF3-06	0.831	0.885	0.879	0.845	0.845	0.828
AF3-07	0.742	0.730	0.724	0.741	0.772	0.807
AF3-08						
AF3-09	0.721	0.739	0.684	0.756	0.729	0.707
AF3-10	0.724	0.714	0.713	0.700	0.725	0.754
AF3-11	0.769	0.847	0.847	0.840	0.849	0.854
AF3-12	0.824	0.808	0.774	0.771	0.774	0.788
AF3-13	0.764	0.763	0.770	0.761	0.794	0.761
AF3-14	0.805	0.819	0.766	0.749	0.800	0.834
Mean	**0.770**	**0.793**	**0.768**	**0.775**	**0.790**	**0.786**
SE	**0.015**	**0.023**	**0.024**	**0.019**	**0.018**	**0.019**

C.3.2. C_{UA} throughout NO_2 exposure sessions

	C_{UA}, (μM)		
Subject	60-pre	post-exposure	60-post
AF3-01	32.57	32.31	20.85
AF3-02			
AF3-03	4.58	5.99	6.32
AF3-04	7.18	6.50	5.53
AF3-05	10.23	16.70	10.90
AF3-06	13.77	11.52	13.47
AF3-07	9.73	14.01	13.77
AF3-08			
AF3-09	9.89	10.65	7.83
AF3-10	11.96	8.28	11.86
AF3-11	11.05	14.26	15.17
AF3-12	21.85	11.96	11.77
AF3-13	19.81	22.80	18.36
AF3-14	17.58	20.43	14.78
Mean	**14.2**	**14.6**	**12.6**
SE	**2.1**	**2.0**	**1.2**

C.3.3. Total Protein Levels throughout NO_2 exposure sessions

	Total Protein, (μg,ml)		
Subject	60-pre	post-exposure	60-post
AF3-01	175.5	240.5	126.5
AF3-02			
AF3-03	98.6	88.8	139.6
AF3-04	198.2	234.4	160.1
AF3-05	116.9	447.4	166.8
AF3-06	147.8	245.1	185.7
AF3-07	123.3	386.1	301.6
AF3-08			
AF3-09	345.2	194.9	249.7
AF3-10	175.5	98.6	275.6
AF3-11	454.9	268.9	220.2
AF3-12	342.1	280.6	260.8
AF3-13	516.2	677.9	550.1
AF3-14	1920.5	1000.5	325.6
Mean	**384.6**	**347.0**	**246.9**
SE	**134.5**	**69.5**	**30.8**

C.3.4. TBARS concentration throughout Air exposure sessions

	TBARS, nM MDA Equivalents		
Subject	60-pre	post-exposure	60-post
N01			
N02			
N03	0.127	0.024	
N04	0.066	0.018	0.188
N05	0.052	0.028	0.033
N06	0.021	0.060	0.016
N07	0.241	0.026	0.009
N08			
N09	0.023	0.055	0.057
N10	0.084		0.022
N11	0.030	0.040	0.027
N12	0.071	0.005	0.152
N14	0.016	0.041	0.027
N15	0.049	0.134	0.031
Mean	**0.071**	**0.043**	**0.056**
SE	**0.020**	**0.011**	**0.020**

C.3.5. TBARS concentration throughout O_3 exposure sessions

	TBARS, nM MDA Equivalents		
Subject	60-pre	post-exposure	60-post
N01	0.049	0.044	0.008
N02			
N03	0.098	0.089	0.050
N04	0.087	0.231	0.202
N05	0.033	0.101	0.105
N06	0.064	0.153	0.078
N07	0.227	0.133	0.077
N09	0.029	0.116	0.238
N10	0.038	0.171	0.130
N11	0.099	0.107	0.127
N12	0.213	0.221	0.219
N14	0.178	0.061	0.249
N15	0.003	0.048	0.124
MEAN	**0.097**	**0.130**	**0.145**
SE	**0.022**	**0.017**	**0.020**

Appendix D: Protocol and Data Analysis for Protocol LS1: Determination of the Contribution of C_{UA} to Λ by Serial Nasal Lavage

The data presented in this section are a result of a protocol design and data acquisition efforts by Lizzie Santiago.

D.1. LS1 Protocol – Determination of the contribution of C_{UA} to Λ by serial nasal lavage

In this study, NLF components were disrupted by performing consecutive nasal lavage maneuvers. It was hypothesized that imposing a series of nasal lavages would subsequently remove uric acid and reduce ozone uptake. This data allows comparison of effects of C_{UA} on Λ within each subject on a one day basis.

Eleven subjects completed the study under IRB and GCRC oversight. Subjects came to the lab on one day for this experiment. Upon clearance by a clinician, subjects underwent measurement of Λ immediately followed by performing nasal lavage. This was repeated every minute for two minutes for a total of three Λ measurements, and nasal lavage samples. The lavage samples were frozen at -80°C, and later analyzed by HPLC for determination of C_{UA}. A timeline of measurements is displayed in figure D.1.

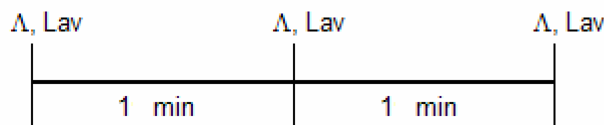

Figure D.1. Timeline of measurements for Protocol LS1.

Data obtained from this experiment are provided in appendix D.2. for each subject. Mean Λ was reduced at each subsequent nasal washing. However, this reduction was only significant after the first washing ($p<0.001$). C_{UA} was significantly reduced following both lavage acquisitions ($p<0.002$).

Figure D.2 displays the relationship between $-\ln(1-\Lambda)$ and $C_{UA}^{1/2}$ for each subject in this study. ANCOVA indicates that $-\ln(1-\Lambda)$ is significantly predicted ($p<0.001$) by subject and $C_{UA}^{1/2}$ as random factor and covariate, respectively. The value for the

slope of this regression was 2978.6 ± 422.5 $l^{1/2}$ $mol^{-1/2}$ which, according to equation 3.14, corresponds to a k_2 value of 6.04 x 10^8 ±.1.71 x 10^8 $M^{-1}s^{-1}$. The intercept of this regression was significantly non-zero ($p<0.001$).

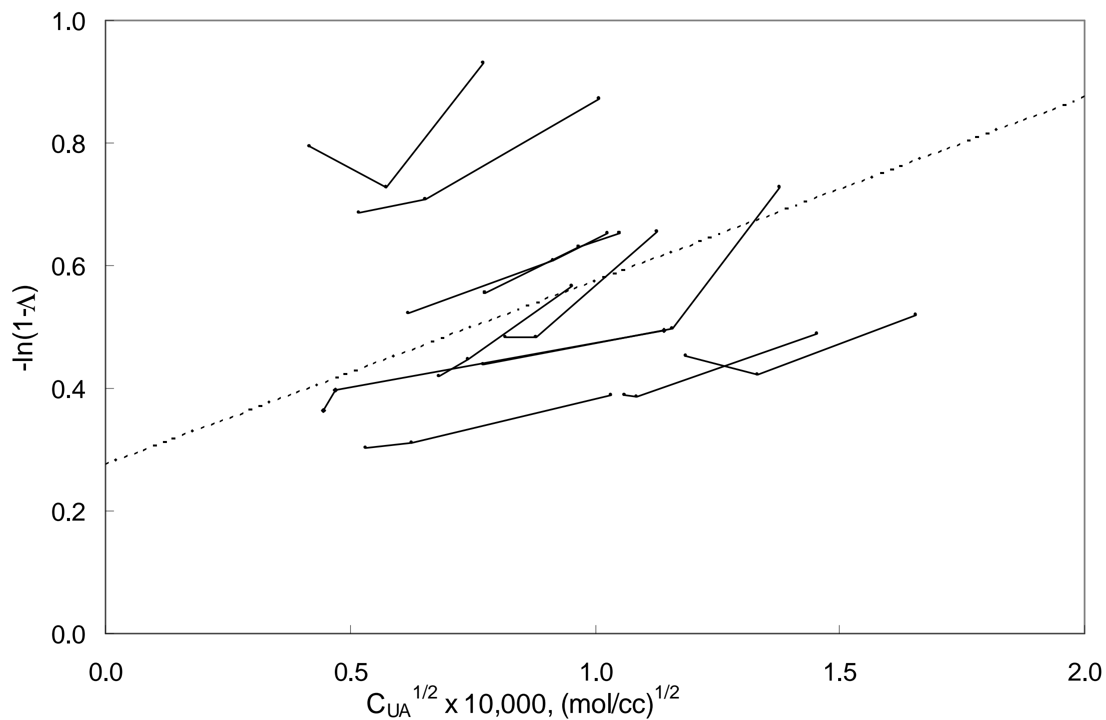

Figure. D.2 Relationship between –ln(1-Λ) and $C_{UA}^{1/2}$ for each subject at each subsequent exposure. Each line connects data obtained for one subject. The dashed line represents the regression obtained by ANOVA which indicated a significant correlation ($p<0.001$).

D.2. Ozone Uptake and C_{UA} at each nasal lavage collection time.

Subject	Λ1	Λ2	Λ3	C_{UA} 1	C_{UA} 2	C_{UA} 3
1	0.390	0.328	0.305	13.1	2.2	2.0
2	0.433	0.361	0.342	9.1	5.5	4.7
3	0.322	0.268	0.261	10.7	3.9	2.8
4	0.405	0.345	0.364	27.4	17.8	14.1
5	0.479	0.456	0.406	10.6	8.4	3.8
6	0.517	0.391	0.355	18.9	13.4	6.0
7	0.481	0.383	0.384	12.7	7.8	6.7
8	0.582	0.507	0.497	10.2	4.3	2.7
9	0.606	0.517	0.548	6.0	3.3	1.7
10	0.386	0.321	0.323	21.2	11.8	11.2
11	0.480	0.467	0.426	11.0	9.3	6.0
Mean	**0.462**	**0.395**	**0.383**	**13.7**	**8.0**	**5.6**
SE	**0.026**	**0.025**	**0.025**	**1.9**	**1.5**	**1.2**

D.3. ANOVA results for protocol LS1

General Linear Model: -ln(1-Λ) versus subject

Factor	Type	Levels	Values
subject	random	11	1 2 3 4 5 6 7 8 9 10 11

Analysis of Variance for -ln(1-Λ), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
subject	10	0.636856	0.749728	0.074973	33.01	0.000
$C_{UA}^{1/2}$	1	0.112871	0.112871	0.112871	49.70	0.000
Error	21	0.047691	0.047691	0.002271		
Total	32	0.797418				

Term	Coef	SE Coef	T	P
Constant	0.27552	0.03908	7.05	0.000
$C_{UA}^{1/2}$	2978.6	422.5	7.05	0.000

Unusual Observations for -ln(1-Λ)

Obs	-ln(1-Λ)	Fit	SE Fit	Residual	St Resid
16	0.727739	0.635769	0.029855	0.091970	2.48R
26	0.727739	0.813462	0.027520	-0.085723	-2.20R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 subject	(3) + 2.8253(1)
2 $C_{UA}^{1/2}$	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 subject	21.00	0.002271	(3)
2 $C_{UA}^{1/2}$	21.00	0.002271	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subject	0.02573
Error	0.00227

Appendix E: Statistical Analysis of Data

E.1. Paired t-test comparisons for AF1

E.1.1. AF1 – Uptake in Air Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

Paired T for pre-exposure - post-exposure

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8141	0.0811	0.0209
post-exposur	15	0.8298	0.0867	0.0224
Difference	15	-0.0157	0.0407	0.0105

95% CI for mean difference: (-0.0382, 0.0069)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.49 P-Value = 0.158

Paired T-Test and CI: pre-exposure, 15-post

Paired T for pre-exposure - 15-post

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8141	0.0811	0.0209
15-post	15	0.8153	0.0921	0.0238
Difference	15	-0.0012	0.0499	0.0129

95% CI for mean difference: (-0.0288, 0.0264)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.09 P-Value = 0.927

Paired T-Test and CI: pre-exposure, 30-post

Paired T for pre-exposure - 30-post

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8141	0.0811	0.0209
30-post	15	0.8213	0.0876	0.0226
Difference	15	-0.0072	0.0437	0.0113

95% CI for mean difference: (-0.0314, 0.0170)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.64 P-Value = 0.534

Paired T-Test and CI: pre-exposure, 60-post

Paired T for pre-exposure - 60-post

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8141	0.0811	0.0209
60-post	15	0.8245	0.0827	0.0213
Difference	15	-0.0104	0.0469	0.0121

95% CI for mean difference: (-0.0364, 0.0156)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.86 P-Value = 0.405

E.1.2. AF1 – Uptake in O_3 Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

Paired T for pre-exposure - post-exposure

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8247	0.0747	0.0193
post-exposur	15	0.7116	0.0830	0.0214
Difference	15	0.1131	0.0506	0.0131

95% CI for mean difference: (0.0851, 0.1411)
T-Test of mean difference = 0 (vs not = 0): T-Value = 8.66 P-Value = 0.000

Paired T-Test and CI: pre-exposure, 15-post

Paired T for pre-exposure - 15-post

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8247	0.0747	0.0193
15-post	15	0.8063	0.0721	0.0186
Difference	15	0.01833	0.03286	0.00848

95% CI for mean difference: (0.00014, 0.03653)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.16 P-Value = 0.049

Paired T-Test and CI: pre-exposure, 30-post

Paired T for pre-exposure - 30-post

	N	Mean	StDev	SE Mean
pre-exposure	15	0.8247	0.0747	0.0193
30-post	15	0.8083	0.0718	0.0185
Difference	15	0.0164	0.0504	0.0130

```
95% CI for mean difference: (-0.0115, 0.0443)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.26  P-Value = 0.228
```

Paired T-Test and CI: pre-exposure, 60-post

```
Paired T for pre-exposure - 60-post

                  N      Mean     StDev   SE Mean
pre-exposure     15    0.8247    0.0747    0.0193
60-post          15    0.8235    0.0690    0.0178
Difference       15   0.00120   0.03289   0.00849

95% CI for mean difference: (-0.01701, 0.01941)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.14  P-Value = 0.890
```

E.1.3. AF1 – C_{NO} throughout Air Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

```
Paired T for pre-exposure - post-exposure

                  N      Mean     StDev   SE Mean
pre-exposure     12    105.93     31.73      9.16
post-exposur     12    111.72     31.60      9.12
Difference       12     -5.79     13.16      3.80

95% CI for mean difference: (-14.15, 2.57)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.52  P-Value = 0.156
```

Paired T-Test and CI: pre-exposure, 15-post

```
Paired T for pre-exposure - 15-post

                  N      Mean     StDev   SE Mean
pre-exposure     12     105.9      31.7       9.2
15-post          12     110.8      34.8      10.0
Difference       12     -4.92     10.37      2.99

95% CI for mean difference: (-11.51, 1.67)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.64  P-Value = 0.128
```

Paired T-Test and CI: pre-exposure, 30-post

```
Paired T for pre-exposure - 30-post

                  N      Mean     StDev   SE Mean
pre-exposure     12    105.93     31.73      9.16
30-post          12    110.80     28.34      8.18
Difference       12     -4.87     10.03      2.90

95% CI for mean difference: (-11.24, 1.50)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.68  P-Value = 0.121
```

Paired T-Test and CI: pre-exposure, 60-post

```
Paired T for pre-exposure - 60-post

                  N      Mean     StDev   SE Mean
pre-exposure     12    105.93     31.73      9.16
60-post          12    110.22     28.51      8.23
Difference       12     -4.30      8.26      2.38

95% CI for mean difference: (-9.54, 0.95)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.80  P-Value = 0.099
```

E.1.4. AF1 – C_{NO} throughout O_3 Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

```
Paired T for pre-exposure - post-exposure

                  N      Mean     StDev   SE Mean
pre-exposure     12    102.72     30.05      8.67
post-exposur     12    107.91     31.31      9.04
Difference       12     -5.19     10.99      3.17

95% CI for mean difference: (-12.18, 1.79)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.64  P-Value = 0.130
```

Paired T-Test and CI: pre-exposure, 15-post

```
Paired T for pre-exposure - 15-post

                  N      Mean     StDev   SE Mean
pre-exposure     12    102.72     30.05      8.67
15-post          12    109.07     31.38      9.06
Difference       12     -6.36     13.03      3.76

95% CI for mean difference: (-14.64, 1.92)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.69  P-Value = 0.119
```

Paired T-Test and CI: pre-exposure, 30-post

```
Paired T for pre-exposure - 30-post

                  N      Mean     StDev   SE Mean
pre-exposure     12    102.72     30.05      8.67
30-post          12    110.53     30.73      8.87
Difference       12     -7.82     10.20      2.95

95% CI for mean difference: (-14.30, -1.33)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.65  P-Value = 0.022
```

Paired T-Test and CI: pre-exposure, 60-post

```
Paired T for pre-exposure - 60-post
```

	N	Mean	StDev	SE Mean
pre-exposure	11	101.06	30.94	9.33
60-post	11	108.69	31.42	9.47
Difference	11	-7.63	10.18	3.07

95% CI for mean difference: (-14.47, -0.79)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.48 P-Value = 0.032

E.1.5. AF1 – NV throughout Air Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

Paired T for pre-exposure - post-exposure

	N	Mean	StDev	SE Mean
pre-exposure	14	56.17	15.94	4.26
post-exposur	14	48.94	11.18	2.99
Difference	14	7.23	9.97	2.66

95% CI for mean difference: (1.47, 12.98)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.71 P-Value = 0.018

Paired T-Test and CI: pre-exposure, 60-post

Paired T for pre-exposure - 60-post

	N	Mean	StDev	SE Mean
pre-exposure	13	57.88	15.18	4.21
60-post	13	53.46	6.45	1.79
Difference	13	4.42	12.46	3.46

95% CI for mean difference: (-3.11, 11.96)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.28 P-Value = 0.225

E.1.6. AF1 – NV throughout O_3 Exposure Sessions

Paired T-Test and CI: pre-exposure, post-exposure

Paired T for pre-exposure - post-exposure

	N	Mean	StDev	SE Mean
pre-exposure	14	55.97	15.56	4.16
post-exposur	14	48.36	7.04	1.88
Difference	14	7.61	14.19	3.79

95% CI for mean difference: (-0.58, 15.81)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.01 P-Value = 0.066

Paired T-Test and CI: pre-exposure, 60-post

Paired T for pre-exposure - 60-post

	N	Mean	StDev	SE Mean
pre-exposure	13	56.34	16.13	4.47
60-post	13	48.98	8.17	2.27
Difference	13	7.35	15.79	4.38

```
95% CI for mean difference: (-2.19, 16.90)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.68  P-Value = 0.119
```

E.2. Paired t-test comparisons for AF2

E.2.1. AF2 – Λ throughout air exposure sessions

Paired T-Test and CI: 60-pre, pre

```
Paired T for 60-pre - pre

                 N      Mean     StDev   SE Mean
60-pre          25     77.79      8.37      1.67
pre             25     79.45      8.93      1.79
Difference      25    -1.658     4.255     0.851

95% CI for mean difference: (-3.415, 0.098)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.95  P-Value = 0.063
```

Paired T-Test and CI: 60-pre, 15-post

```
Paired T for 60-pre - 15-post

                 N      Mean     StDev   SE Mean
60-pre          25     77.79      8.37      1.67
15-post         25     77.54      8.78      1.76
Difference      25      0.25      5.70      1.14

95% CI for mean difference: (-2.10, 2.61)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.22  P-Value = 0.828
```

Paired T-Test and CI: 60-pre, post

```
Paired T for 60-pre - post

                 N      Mean     StDev   SE Mean
60-pre          25     77.79      8.37      1.67
post            25     79.69      9.22      1.84
Difference      25     -1.90      5.82      1.16

95% CI for mean difference: (-4.30, 0.50)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.63  P-Value = 0.116
```

Paired T-Test and CI: 60-pre, 15-post

```
Paired T for 60-pre - 15-post

                 N      Mean     StDev   SE Mean
60-pre          25     77.79      8.37      1.67
15-post         25     77.54      8.78      1.76
Difference      25      0.25      5.70      1.14
```

95% CI for mean difference: (-2.10, 2.61)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.22 P-Value = 0.828

Paired T-Test and CI: 60-pre, 30-post

Paired T for 60-pre - 30-post

	N	Mean	StDev	SE Mean
60-pre	25	77.79	8.37	1.67
30-post	25	78.30	8.81	1.76
Difference	25	-0.51	5.69	1.14

95% CI for mean difference: (-2.86, 1.84)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.45 P-Value = 0.656

Paired T-Test and CI: 60-pre, 60-post

Paired T for 60-pre - 60-post

	N	Mean	StDev	SE Mean
60-pre	25	77.79	8.37	1.67
60-post	25	79.58	8.28	1.66
Difference	25	-1.797	4.570	0.914

95% CI for mean difference: (-3.683, 0.089)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.97 P-Value = 0.061

Paired T-Test and CI: pre, post

Paired T for pre - post

	N	Mean	StDev	SE Mean
pre	25	79.45	8.93	1.79
post	25	79.69	9.22	1.84
Difference	25	-0.241	4.389	0.878

95% CI for mean difference: (-2.053, 1.570)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.27 P-Value = 0.786

Paired T-Test and CI: pre, 15-post

Paired T for pre - 15-post

	N	Mean	StDev	SE Mean
pre	25	79.45	8.93	1.79
15-post	25	77.54	8.78	1.76
Difference	25	1.910	4.243	0.849

95% CI for mean difference: (0.158, 3.661)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.25 P-Value = 0.034

Paired T-Test and CI: pre, 30-post

Paired T for pre - 30-post

	N	Mean	StDev	SE Mean
pre	25	79.45	8.93	1.79
30-post	25	78.30	8.81	1.76
Difference	25	1.145	3.816	0.763

95% CI for mean difference: (-0.430, 2.720)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.50 P-Value = 0.147

Paired T-Test and CI: pre, 60-post

Paired T for pre - 60-post

	N	Mean	StDev	SE Mean
pre	25	79.45	8.93	1.79
60-post	25	79.58	8.28	1.66
Difference	25	-0.139	3.718	0.744

95% CI for mean difference: (-1.673, 1.396)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.19 P-Value = 0.853

E.2.2. AF2 – Λ throughout O_3 exposure sessions

Paired T-Test and CI: 60-pre, pre

Paired T for 60-pre - pre

	N	Mean	StDev	SE Mean
60-pre	24	0.7738	0.0839	0.0171
pre	24	0.7988	0.0935	0.0191
Difference	24	-0.0250	0.0603	0.0123

95% CI for mean difference: (-0.0505, 0.0004)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.03 P-Value = 0.054

Paired T-Test and CI: 60-pre, post

Paired T for 60-pre - post

	N	Mean	StDev	SE Mean
60-pre	24	0.7738	0.0839	0.0171
post	24	0.6481	0.0908	0.0185
Difference	24	0.1257	0.0883	0.0180

95% CI for mean difference: (0.0884, 0.1630)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.97 P-Value = 0.000

Paired T-Test and CI: 60-pre, 15-post

Paired T for 60-pre - 15-post

	N	Mean	StDev	SE Mean
60-pre	24	0.7738	0.0839	0.0171
15-post	24	0.7720	0.1021	0.0208
Difference	24	0.0018	0.0637	0.0130

95% CI for mean difference: (-0.0251, 0.0287)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.14 P-Value = 0.890

Paired T-Test and CI: 60-pre, 30-post

Paired T for 60-pre - 30-post

	N	Mean	StDev	SE Mean
60-pre	24	0.7738	0.0839	0.0171
30-post	24	0.7943	0.1023	0.0209
Difference	24	-0.0205	0.0608	0.0124

95% CI for mean difference: (-0.0462, 0.0051)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.65 P-Value = 0.112

Paired T-Test and CI: 60-pre, 60-post

Paired T for 60-pre - 60-post

	N	Mean	StDev	SE Mean
60-pre	23	0.7714	0.0850	0.0177
60-post	23	0.7939	0.0942	0.0196
Difference	23	-0.0225	0.0655	0.0137

95% CI for mean difference: (-0.0509, 0.0058)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.65 P-Value = 0.113

Paired T-Test and CI: pre, 15-post

Paired T for pre - 15-post

	N	Mean	StDev	SE Mean
pre	24	0.7988	0.0935	0.0191
15-post	24	0.7720	0.1021	0.0208
Difference	24	0.02682	0.04567	0.00932

95% CI for mean difference: (0.00754, 0.04610)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.88 P-Value = 0.009

Paired T-Test and CI: pre, 30-post

Paired T for pre - 30-post

	N	Mean	StDev	SE Mean

	N	Mean	StDev	SE Mean
pre	24	0.7988	0.0935	0.0191
30-post	24	0.7943	0.1023	0.0209
Difference	24	0.00448	0.04015	0.00820

95% CI for mean difference: (-0.01247, 0.02144)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.55 P-Value = 0.590

Paired T-Test and CI: pre, 60-post

Paired T for pre - 60-post

	N	Mean	StDev	SE Mean
pre	23	0.7978	0.0955	0.0199
60-post	23	0.7939	0.0942	0.0196
Difference	23	0.0039	0.0519	0.0108

95% CI for mean difference: (-0.0185, 0.0263)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.36 P-Value = 0.723

E.2.3. AF2 – C_{UA} throughout air exposure sessions

AF2 – C_{UA} in Air Exposure Sessions

Paired T-Test and CI: 60-pre, post-exposure

Paired T for 60-pre - post-exposure

	N	Mean	StDev	SE Mean
60-pre	24	18.48	10.62	2.17
post-exposur	24	20.83	10.20	2.08
Difference	24	-2.36	4.92	1.00

95% CI for mean difference: (-4.44, -0.28)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.35 P-Value = 0.028

Paired T-Test and CI: 60-pre, 60-post

Paired T for 60-pre - 60-post

	N	Mean	StDev	SE Mean
60-pre	24	18.48	10.62	2.17
60-post	24	18.13	12.08	2.47
Difference	24	0.35	8.41	1.72

95% CI for mean difference: (-3.20, 3.90)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.20 P-Value = 0.840

E.2.4. AF2 – C_{UA} throughout O_3 exposure sessions

Paired T-Test and CI: 60-pre, post-exposure

```
Paired T for 60-pre - post-exposure

                  N      Mean     StDev   SE Mean
60-pre           24     19.18     10.27      2.10
post-exposur     24     13.40     10.03      2.05
Difference       24     5.779     4.715     0.962

95% CI for mean difference: (3.788, 7.770)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.00  P-Value = 0.000
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                  N      Mean     StDev   SE Mean
60-pre           24     19.18     10.27      2.10
60-post          24     17.50     11.83      2.41
Difference       24      1.68      7.52      1.53

95% CI for mean difference: (-1.49, 4.86)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.10  P-Value = 0.284
```

E.2.5. AF2 – Protein levels throughout air exposure sessions

Paired T-Test and CI: 60-pre, Post

```
Paired T for 60-pre - Post

                  N      Mean     StDev   SE Mean
60-pre           21     207.5      62.6      13.7
Post             21     203.4      71.7      15.7
Difference       21       4.1      64.7      14.1

95% CI for mean difference: (-25.4, 33.5)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.29  P-Value = 0.777
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                  N      Mean     StDev   SE Mean
60-pre           21     207.5      62.6      13.7
60-post          21     195.0      56.3      12.3
Difference       21      12.5      72.7      15.9

95% CI for mean difference: (-20.6, 45.6)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.79  P-Value = 0.440
```

E.2.6. AF2 – Protein levels throughout O_3 exposure sessions

Paired T-Test and CI: 60-pre, post

```
Paired T for 60-pre - post

                  N      Mean     StDev   SE Mean
60-pre           21     209.1      57.8      12.6
post             21     212.2      62.2      13.6
Difference       21     -3.12     39.22      8.56

95% CI for mean difference: (-20.97, 14.73)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.36  P-Value = 0.719
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                  N      Mean     StDev   SE Mean
60-pre           21     209.1      57.8      12.6
60-post          21     187.5      44.3       9.7
Difference       21      21.6      47.6      10.4

95% CI for mean difference: (-0.1, 43.2)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.08  P-Value = 0.051
```

E.3. Paired t-test comparisons for AF3

E.3.1. AF3 – Λ throughout NO_2 exposure sessions

Paired T-Test and CI: 60-pre, Pre

```
Paired T for 60-pre - Pre

                  N      Mean     StDev   SE Mean
60-pre           12    0.7700    0.0554    0.0160
Pre              12    0.7927    0.0846    0.0244
Difference       12   -0.0227    0.0419    0.0121

95% CI for mean difference: (-0.0493, 0.0040)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.87  P-Value = 0.088
```

Paired T-Test and CI: 60-pre, Post

```
Paired T for 60-pre - Post

                  N      Mean     StDev   SE Mean
60-pre           12    0.7700    0.0554    0.0160
Post             12    0.7678    0.0911    0.0263
Difference       12    0.0022    0.0544    0.0157

95% CI for mean difference: (-0.0323, 0.0368)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.14  P-Value = 0.889
```

Paired T-Test and CI: 60-pre, 15-post

```
Paired T for 60-pre - 15-post
```

	N	Mean	StDev	SE Mean
60-pre	12	0.7700	0.0554	0.0160
15-post	12	0.7751	0.0708	0.0204
Difference	12	-0.0051	0.0407	0.0118

95% CI for mean difference: (-0.0310, 0.0208)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.43 P-Value = 0.674

Paired T-Test and CI: 60-pre, 30-post

Paired T for 60-pre - 30-post

	N	Mean	StDev	SE Mean
60-pre	12	0.7700	0.0554	0.0160
30-post	12	0.7897	0.0670	0.0193
Difference	12	-0.0197	0.0349	0.0101

95% CI for mean difference: (-0.0418, 0.0025)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.95 P-Value = 0.077

Paired T-Test and CI: 60-pre, 60-post

Paired T for 60-pre - 60-post

	N	Mean	StDev	SE Mean
60-pre	12	0.7700	0.0554	0.0160
60-post	12	0.7857	0.0729	0.0210
Difference	12	-0.0157	0.0412	0.0119

95% CI for mean difference: (-0.0418, 0.0105)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.32 P-Value = 0.215

Paired T-Test and CI: Pre, Post

Paired T for Pre - Post

	N	Mean	StDev	SE Mean
Pre	12	0.7927	0.0846	0.0244
Post	12	0.7678	0.0911	0.0263
Difference	12	0.0249	0.0391	0.0113

95% CI for mean difference: (0.0001, 0.0498)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.21 P-Value = 0.049

Paired T-Test and CI: Pre, 15-post

Paired T for Pre - 15-post

	N	Mean	StDev	SE Mean
Pre	12	0.7927	0.0846	0.0244

	N	Mean	StDev	SE Mean
15-post	12	0.7751	0.0708	0.0204
Difference	12	0.01758	0.02600	0.00751

95% CI for mean difference: (0.00106, 0.03410)
T-Test of mean difference = 0 (vs not = 0): T-Value = 2.34 P-Value = 0.039

Paired T-Test and CI: Pre, 30-post

Paired T for Pre - 30-post

	N	Mean	StDev	SE Mean
Pre	12	0.7927	0.0846	0.0244
30-post	12	0.7897	0.0670	0.0193
Difference	12	0.00300	0.02743	0.00792

95% CI for mean difference: (-0.01443, 0.02043)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.38 P-Value = 0.712

Paired T-Test and CI: Pre, 60-post

Paired T for Pre - 60-post

	N	Mean	StDev	SE Mean
Pre	12	0.7927	0.0846	0.0244
60-post	12	0.7857	0.0729	0.0210
Difference	12	0.0070	0.0439	0.0127

95% CI for mean difference: (-0.0209, 0.0349)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.55 P-Value = 0.592

E.3.2. AF3 – C_{UA} throughout NO_2 exposure sessions

Paired T-Test and CI: 60-pre, post-exposure

Paired T for 60-pre - post-exposure

	N	Mean	StDev	SE Mean
60-pre	12	14.18	7.69	2.22
post-exposur	12	14.62	7.58	2.19
Difference	12	-0.43	4.31	1.25

95% CI for mean difference: (-3.18, 2.31)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.35 P-Value = 0.734

Paired T-Test and CI: 60-pre, 60-post

Paired T for 60-pre - 60-post

	N	Mean	StDev	SE Mean
60-pre	12	14.18	7.69	2.22
60-post	12	12.55	4.58	1.32
Difference	12	1.63	4.87	1.41

```
95% CI for mean difference: (-1.46, 4.73)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.16  P-Value = 0.270
```

AF3 – Protein in NO2 Exposure Sessions

Paired T-Test and CI: 60-pre, post-exposure

```
Paired T for 60-pre - post-exposure

                 N      Mean     StDev   SE Mean
60-pre          11     244.9     145.3      43.8
post-exposur    11     287.6     166.9      50.3
Difference      11     -42.6     163.2      49.2

95% CI for mean difference: (-152.3, 67.0)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.87  P-Value = 0.407
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                 N      Mean     StDev   SE Mean
60-pre          11     244.9     145.3      43.8
60-post         11     239.7     118.2      35.6
Difference      11       5.2     110.9      33.4

95% CI for mean difference: (-69.3, 79.7)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.16  P-Value = 0.879
```

E.3.3. AF3 – TBARS levels throughout air exposure sessions

Paired T-Test and CI: 60-pre, post-exposure

```
Paired T for 60-pre - post-exposure

                 N      Mean     StDev   SE Mean
60-pre          10    0.0696    0.0686    0.0217
post-exposur    10    0.0431    0.0360    0.0114
Difference      10    0.0265    0.0866    0.0274

95% CI for mean difference: (-0.0355, 0.0885)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.97  P-Value = 0.359
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                 N      Mean     StDev   SE Mean
60-pre          10    0.0653    0.0659    0.0208
60-post         10    0.0562    0.0619    0.0196
Difference      10    0.0091    0.0944    0.0299

95% CI for mean difference: (-0.0585, 0.0767)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.30  P-Value = 0.768
```

E.3.4. AF3 – TBARS levels throughout O_3 exposure sessions

Paired T-Test and CI: 60-pre, post-exposure

```
Paired T for 60-pre - post-exposure

                  N      Mean     StDev   SE Mean
60-pre           10    0.0713    0.0591    0.0187
post     10    0.1281    0.0650    0.0206
Difference       10   -0.0568    0.0565    0.0179

95% CI for mean difference: (-0.0972, -0.0163)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.17  P-Value = 0.011
```

Paired T-Test and CI: 60-pre, 60-post

```
Paired T for 60-pre - 60-post

                  N      Mean     StDev   SE Mean
60-pre           10    0.0713    0.0591    0.0187
60-post          10    0.1280    0.0740    0.0234
Difference       10   -0.0567    0.0800    0.0253

95% CI for mean difference: (-0.1139, 0.0006)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.24  P-Value = 0.052
```

E.4. Analysis of Covariance for –ln(1-Λ) vs Subject, $C_{UA}^{1/2}$

E.4.1 Day to day

General Linear Model: -ln(1-Λ) versus subj

```
Factor     Type Levels Values
subj     random     23  1  2  3  4  5  6  7  9 10 11 12 14 15 16 17 18 19 20
21
                       22 23 24 25

Analysis of Variance for -ln(1-L), using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
subj       22    8.91093    8.67979    0.39454    6.47  0.000
CUA 1/2     1    0.11448    0.11448    0.11448    1.88  0.185
Error      22    1.34204    1.34204    0.06100
Total      45   10.36744

Term          Coef   SE Coef         T      P
Constant    1.0896    0.3763      2.90  0.008
CUA 1/2       3907      2852      1.37  0.185

Unusual Observations for -ln(1-Λ)

Obs  -ln(1-Λ)       Fit      SE Fit  Residual   St Resid
  5   3.49551   2.88032     0.17775   0.61519      3.59R
 29   2.17449   2.78967     0.17775  -0.61519     -3.59R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source       Expected Mean Square for Each Term
 1 subj      (3) +  1.9214(1)
 2 CUA 1/2    (3) + Q[2]
 3 Error     (3)

Error Terms for Tests, using Adjusted SS

Source       Error DF  Error MS  Synthesis of Error MS
 1 subj         22.00   0.06100  (3)
 2 CUA 1/2       22.00   0.06100  (3)

Variance Components, using Adjusted SS

Source    Estimated Value
subj              0.17359
Error             0.06100
```

E.4.2 Pre-post Air Exposure

General Linear Model: -ln(1-Λ) versus subj

Factor	Type	Levels	Values
subj	random	25	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Analysis of Variance for -ln(1-Λ), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
subj	24	14.29458	12.97417	0.54059	11.92	0.000
$C_{UA}^{1/2}$	1	0.15576	0.15576	0.15576	3.44	0.076
Error	24	1.08811	1.08811	0.04534		
Total	49	15.53845				

Term	Coef	SE Coef	T	P
Constant	1.0080	0.3566	2.83	0.009
$C_{UA}^{1/2}$	4711	2542	1.85	0.076

Unusual Observations for -ln(1-Λ)

Obs	-ln(1-Λ)	Fit	SE Fit	Residual	St Resid
14	1.57988	1.96919	0.15092	-0.38931	-2.59R
39	2.39690	2.00758	0.15092	0.38931	2.59R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 subj	(3) + 1.9233(1)
2 $C_{UA}^{1/2}$	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 subj	24.00	0.04534	(3)
2 $C_{UA}^{1/2}$	24.00	0.04534	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subj	0.25750
Error	0.04534

E.4.3 Pre-Post O_3 Exposure

General Linear Model: -ln(1-Λ) versus Subject

```
Factor     Type Levels Values
Subject  random     24  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19
                       20 21 22 23 24
```

Analysis of Variance for -ln(1-Λ), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Subject	23	3.8685	5.4701	0.2378	1.95	0.058
$C_{UA}^{1/2}$	1	2.1530	2.1530	2.1530	17.69	0.000
Error	23	2.8000	2.8000	0.1217		
Total	47	8.8215				

Term	Coef	SE Coef	T	P
Constant	-0.3057	0.3902	-0.78	0.441
$C_{UA}^{1/2}$	13376	3181	4.21	0.000

Unusual Observations for -ln(1-Λ)

Obs	-ln(1-Λ)	Fit	SE Fit	Residual	St Resid
24	2.68336	1.98941	0.25103	0.69394	2.86R
48	0.90552	1.59946	0.25103	-0.69394	-2.86R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 Subject	(3) + 1.9277(1)
2 $C_{UA}^{1/2}$	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 Subject	23.00	0.1217	(3)
2 $C_{UA}^{1/2}$	23.00	0.1217	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
Subject	0.06022
Error	0.12174

E.4.4 Pre Post NO_2

General Linear Model: -ln(1-Λ) versus Subj

Factor	Type	Levels	Values
Subj	random	12	1 2 3 4 5 6 7 8 9 10 11 12

Analysis of Variance for -ln(1-Λ), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Subj	11	1.79731	1.55693	0.14154	6.07	0.003
$C_{UA}^{1/2}$	1	0.07279	0.07279	0.07279	3.12	0.105
Error	11	0.25639	0.25639	0.02331		
Total	23	2.12649				

Term	Coef	SE Coef	T	P
Constant	0.7730	0.4182	1.85	0.092
$C_{UA}^{1/2}$	6328	3581	1.77	0.105

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 Subj	(3) + 1.8346(1)
2 $C_{UA}^{1/2}$	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 Subj	11.00	0.02331	(3)
2 $C_{UA}^{1/2}$	11.00	0.02331	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
Subj	0.06445
Error	0.02331

E.5.1 ANOVA: Λ-NV

General Linear Model: L versus subject

Factor	Type	Levels	Values
subject	random	14	2 11 12 13 15 21 25 26 27 28 29 30 31 33

Analysis of Variance for L, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
subject	13	2098.02	1743.70	134.13	2.84	0.011
NV	1	69.54	69.54	69.54	1.47	0.236
Error	26	1226.12	1226.12	47.16		
Total	40	3393.69				

Term	Coef	SE Coef	T	P
Constant	69.639	7.132	9.76	0.000
NV	0.1673	0.1378	1.21	0.236

Unusual Observations for L

Obs	L	Fit	SE Fit	Residual	St Resid
26	49.1300	60.6015	3.9785	-11.4715	-2.05R
28	71.7300	84.3782	4.0158	-12.6482	-2.27R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 subject	(3) + 2.8090(1)
2 NV	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 subject	26.00	47.16	(3)
2 NV	26.00	47.16	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subject	30.96
Error	47.16

E.5.2 ANOVA: NO-NV

General Linear Model: NO versus subject

Factor Type Levels Values
subject random 12 11 12 13 15 21 26 27 28 29 30 31
32

Analysis of Variance for NO, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
subject	11	19860.1	15232.5	1384.8	12.92	0.000
NV	1	28.4	28.4	28.4	0.26	0.617
Error	11	1178.6	1178.6	107.1		
Total	23	21067.0				

Term	Coef	SE Coef	T	P
Constant	114.08	19.09	5.98	0.000
NV	-0.1766	0.3432	-0.51	0.617

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 subject	(3) + 1.8470(1)
2 NV	(3) + Q[2]
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 subject	11.00	107.1	(3)
2 NV	11.00	107.1	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subject	691.7
Error	107.1

E.5.3 ANOVA: Λ-NO

General Linear Model: U1 versus subj

Factor	Type	Levels	Values
subj	random	14	1 2 3 4 5 6 7 9 10 11 12 13 14 15

Analysis of Variance for U1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
NO1	1	9.61	26.12	26.12	3.29	0.093
subj	13	1530.99	1530.99	117.77	14.82	0.000
Error	13	103.33	103.33	7.95		
Total	27	1643.93				

Term	Coef	SE Coef	T	P
Constant	72.733	4.893	14.86	0.000
NO1	0.08068	0.04450	1.81	0.093

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 NO1	(3) + Q[1]
2 subj	(3) + 1.8662(2)
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 NO1	13.00	7.95	(3)
2 subj	13.00	7.95	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subj	58.847
Error	7.948

E.5.4 ANOVA: Λ-Protein

General Linear Model: Λ versus subj

Factor	Type	Levels	Values
subj	random	20	1 2 3 4 5 6 7 8 9 10 12 14 16 17 19 20 21 22 23 25

Analysis of Variance for UO, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
P	1	70.52	34.99	34.99	1.48	0.239
subj	19	2690.63	2690.63	141.61	5.97	0.000
Error	19	450.59	450.59	23.72		
Total	39	3211.74				

Term	Coef	SE Coef	T	P
Constant	74.655	4.988	14.97	0.000
P	0.02801	0.02306	1.21	0.239

Unusual Observations for Λ

Obs	Λ	Fit	SE Fit	Residual	St Resid
14	79.9700	88.9706	3.4934	-9.0006	-2.65R
20	91.9700	84.9196	3.6868	7.0504	2.22R
34	99.4000	90.3994	3.4934	9.0006	2.65R
40	74.6700	81.7204	3.6868	-7.0504	-2.22R

R denotes an observation with a large standardized residual.

Expected Mean Squares, using Adjusted SS

Source	Expected Mean Square for Each Term
1 P	(3) + Q[1]
2 subj	(3) + 1.9152(2)
3 Error	(3)

Error Terms for Tests, using Adjusted SS

Source	Error DF	Error MS	Synthesis of Error MS
1 P	19.00	23.72	(3)
2 subj	19.00	23.72	(3)

Variance Components, using Adjusted SS

Source	Estimated Value
subj	61.56
Error	23.72

Vita

Ali Fassih

Education:

Ph.D. Chemical Engineering, May 2007. The Pennsylvania State University
B.S. Chemical Engineering, August 2000. The Pennsylvania State University

Research Experience:

The Pennsylvania State University – Department of Chemical Engineering
Ph.D. Thesis Topic: *Ozone uptake in the Human Nasal Cavity: The Role of Uric Acid*
• Designed and implemented clinical studies to investigate the absorption of ozone in the human airways and identify biochemical and physiological responses to ozone exposure.
• Implemented a mathematical model to understand the reactive diffusion of ozone in the human airways

Johnson & Johnson - ALZA Corporation (Mountain View, CA)
Biomedical Engineering Graduate Intern, June 2004-December 2004
• Performed technical and market feasibility studies for five self generated medical device concepts
• Driver of two medical device prototype development studies leading to two invention records

Teaching Experience:

The Pennsylvania State University – Department of Chemical Engineering
Teaching Assistant. 2001-2005
• Assisted professors in teaching classes for seven semesters in the following courses: fluid mechanics, heat transfer, phase and chemical equilibria, advanced reactor design, and chemical process control
• Duties included: Teaching lectures, holding weekly review of class materials, designing questions for projects and homework, holding weekly office hours

The Princeton Review
Instructor for MCAT preparation course, January 2004-Present
• Teach intensive courses in general chemistry, organic chemistry, and physics relevant to the MCAT exam

Presentations

• Biomedical Engineering Society Annual Meeting, Poster Presentation, Philadelphia, PA 2004
• Penn State University Biomolecular Transport Dynamics Symposium, Poster Presentation 2004
• Penn State University Life Sciences Consortium Graduate Research Fellowship, 2000